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ABSTRACT

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its antitumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy. In trying to understand the mechanism underlying E1A's antitumor activity, we have found that E1A downregulated VEGF expression both *in vitro* and *in vivo*, and mapped the domains required for this activity. We have also identified additional new target genes that were critically involved in E1A–mediated chemosensitization and tumor suppression. In addition, I am planning to wire the panoramic signaling networks associated with E1A by systems biology approaches utilizing the genomic and proteomic technologies in an inducible E1A expression model system. These studies can provide useful information for us to better understand the molecular functions of E1A, and hopefully we can use this knowledge to better design a mutant E1A construct for cancer gene therapy in the future.

Table of Contents

Introduction:

The purpose of this project is to study the molecular mechanisms underlying E1A's pro-apoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its anti-tumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and phase II clinical trials are undergoing, we therefore plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effect for cancer gene therapy in the future. The successful reconstruction of this mutant E1A gene will largely depend on our studies of the different mutant stable cells *in vitro* and *in vivo*.

Body

Statement of Work (no change):

Task 1: To test whether E1A could repress VEGF expression and whether or not mutation of the CR1 domain of E1A will abrogate this activity.

Task 2: To test whether CR1 domain of E1A represses VEGF transcription through recruitment of HDAC-1 via binding with p300

Task 3: To test whether E1A promotes apoptosis through its CR2 domain by disruption pRB-E2F-1 complexes and releasing free E2F-1.

Task 4: To test the therapeutic efficacy of the E1A N-terminal mutant as an alternative construct for gene therapy in an orthotopic breast cancer model in nude mouse.

A. Studies and Results:

In the past four years, we have continued to work on understanding the molecular mechanisms underlying E1A's pro-apoptotic effect and anti-tumor activity. In addition, we have been trying to identify additional molecules that may be regulated by E1A via the genomic and proteomic

approaches. This work may provide useful information for identification of novel target genes of E1A and new biomarkers as therapeutic targets. The progress for each aim will be discussed separately as following:

Task 1:

We have observed that stable expression of E1A do repress VEGF expression by Northen blot in cell culture *in vitro* (**Figure 1A**) and by immunohistochemistry in tumor tissue *in vivo* (**Figure 1B**). We also mapped the domains of E1A responsible for downregualtion of VEGF expression by Western blot (**Figure 1C**). We found that deletion mutation of an N-terminal fragment (ΔNT) of E1A enhanced the repression effect of E1A in VEGF expression in animal models (**Figure 1C**). However, deletion either CR1 or CR2 or double deletion of both N-terminal and CR2 domains comprised E1A's function in repression VEGF expression (**Figure 1C**).

Task 2:

Using antibody array approach, we identified quite a few molecules that are associated with E1A (**Figure 2 A-C**). By comparing the antibody array pattern obtained from cell lysates of wild type E1A and different functional domain deletion mutants of E1A, we obtained evidences to support our hypothesis that repression of VEGF expression by E1A is achieved by inhibition of the histone accetyltransferase (HAT) activity of p300 via HDAC1. We found that the mutation of these functional domain do affect E1A's association with several proteins (**Figure 3A**). In particular, the N-terminal deletion mutant binds with HDAC1 more than wild-type E1A, while deletion of either the N-terminal or CR1 domain did not affect E1A binding with pRB or pRB2. However, deletion mutation of the CR2 domain resulted in a loss of binding with pRB, though its binding with pRB1 was enhanced (**Figure 3A**). Because wild-type E1A and the CR2 mutant bind HDAC1 to a lesser extent, the binding of E1A with HDAC1 could be through indirect binding with pRB. In addition, to test whether the altered binding with HDAC1 in ΔNT mutant will contribute to its enhanced activity in repressing the expression of VEGF through p300, we transiently transfected wild-type E1A and different domain deletion mutant stable cells with a Flag-tagged HADC1 construct and did a co-IP study by IP with anti-Flag antibody (M2) and Western blot with p300. We found that more p300 were recruited to HDAC1 in ΔNT cells than the wild type E1A or ΔCR1 or ΔCR2 mutant (**Figure 3B**). These findings consistent with our studies on VEGF expression in tumor tissues obtained from animals inoculated with either wild type E1A or different mutant E1A stable

cells (**Figure 1C**) and strongly suggest that repression of VEGF expression by E1A might be achieved by inhibition of the histone acetyltransferase (HAT) activity of p300 through its binding with pRB and recruitment of HDAC1.

Task3:

In the past four years, we have been intensively working on the molecular mechanisms underlying E1A's pro-apoptotic effect. We have not yet been able to justify the role of free E2F-1in E1A–mediated chemosensitization, however, report from Dr. Lowe's group did show that free E2F-1 played a critical role in E1A-mediated chemosensitization in normal fibroblasts through up-regulation of pro-caspase enzymes, such as caspase-3, -7, -8, and –9 (**Figure 4**, adopted from Nahle Z, et al, ***Nature Cell Biology***, 2002; 4 (11): 859 – 864) (1). We then screened our E1A stable cell lines established in human cancer cells with epithelial origin, such as human breast (MCF-7, MDA-MB-231), ovarian (2774, SKOV3.ip1), and prostate cancer cell lines (PC-3) and we did not obtain a consensus enhancement of these procaspase enzymes (**Figure 5A**). In addition, loss of pRB expression in human cancer cells either from epithelial origin or from fibroblast did not result in enhanced expression of these procaspase enzymes (**Figure 5B**). These results suggest that additional mechanisms may be involved in E1A-mediated sensitization to drug-induced apoptosis in human cancer cells.

Therefore, in addition to the original proposed experiment, we also explored additional molecules involved in the regulation of apoptosis and we found that E1A downregulated a critical oncogenic survival factor Akt while upregulated a proapoptotic factor p38. We demonstrated that activation of p38 and inactivation of Akt were necessary and sufficient for E1A–mediated sensitization to apoptosis induced by serum-starvation, ultraviolet (UV) -irradiation, tumor necrosis factor (TNF) -α, and different categories of anti-cancer drugs, such as adriamycin/doxorubicin, cisplatin, methotrexate, gemcitabine and paclitaxel (Taxol) (data published in ***MCB***, 2003,23: 6836-48, also see appendix). Further studies show that E1A by upregulation of the catalytic subunit of PP2A (PP2A/C) enhanced the activity of PP2A, which results in repression of Akt activation in E1A expressing cells (**Figure 6 A-B**). In addition, we showed that activation of PP2A/C is required for E1A–mediated sensitization to drug–induced apoptosis, since blocking PP2A/C expression using a specific small interfering RNA (siRNA) against PP2A/C reduced drug sensitivity in E1A-expressing cells. Deletion mutation of the CR2 of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, also abolished E1A's ability to upregulate PP2A/C (a paper summarizing this results have been published in ***Cancer Research*** as **Advance in Brief**, 2004,

64:5938-42, see appendix). A comprehensive review in a book chapter to be published by Landes Bioscience summarized these findings and discussed their implications in detail (see appendix).

Recent reports published in ***Nature Genetics*** suggested a tumor suppressor role for p38, as inactivation of p38 by a protein phosphatase PPM1D (also called Wip1, which stands for a wild-type p53-inducible protein phosphatase) is involved in the development of human cancers by suppressing p53 activation (2-4). Since Wip1 is also located within a breast cancer amplification epicenter (5), we wanted to test if E1A could affect Wip1 activity. By screening several pairs of E1A stable cell lines established in human breast cancer, we found that the expression of E1A did repress Wip1 protein expression when compared with the respective vector transfected control cells (**Figure 7**). In addition, we also found that repression of Wip1 expression was correlated with E1A-mediated activation of p38 activity and inactivation of Akt (**Figure 7**). By using small interference RNA (siRNA) approach, we blocked Wip1 expression and observed that repression of Wip1 also resulted in enhanced expression of phosphorylated p38 (**Figure 8A**) and increased spontaneous apoptosis in MCF-7 cells after blockade of Wip1 by the siRNA (**Figure 8B**). Therefore, we constructed a set of retroviral vectors expressing stable Wip1 siRNA against various domains of Wip1 and were transfected into MCF-7 cells. Stable cells with reduced levels of Wip1 expression were selected and ready for future characterization. As Wip1 is also located within a breast cancer amplification epicenter (5), we therefore proposed to determine if Akt will directly affect Wip1 phosphatase activity and indirectly affect p38 activation. We found that Akt directly associated with Wip1 (**Figure 9A**) and phosphorylated Wip1 by *in vitro* kinase assay using purified recombinant GST-Wip1 as a substrate for Akt (**Figure 9B**). Further experiments are proposed to determine which site(s) of Wip1 will be phosphorylated by Akt and whether phosphorylation of Wip1 by Akt affects Wip1 phosphatase activity toward its substrates, such as p53 and p38. To better understand the biological function of Wip1 and map the domain(s) interacts with Akt, a serial deletion mutation constructs of Wip1 were established. In addition, to further evaluate the role of Wip1 in mammary tumorigenesis, a mammary specific Wip1 overexpression transgenic model is planned. We will also validate whether Wip1 is a good target for the treatment of human breast cancer by in vitro as well as in vivo studies using the Wip1 transgenic model. A grant proposal for these studies has been successfully founded by the Susan G. Komen Breast Cancer Foundation (see appendix). Once the Wip1 transgenic model is available, I will plan to screen against a small molecule compound library and or peptide library to find specific Wip1 inhibitor for potential translational application in the future.

Task 4:

The results we obtained from the animal model study showed that at least the N-terminal mutant of E1A worked as good as that of the wild-type E1A in terms of repressing tumor growth and sensitization to chemotherapy *in vivo* in the context of a systemic gene therapy (**Figure 10**). After combined two independent experiments together (N= 8), we monitored the survival rate of wild-type E1A or N-terminal deletion mutant with or without combination with Taxol for more than a year. Again, we observed that the N-terminal mutant of E1A worked as good as that of the wild-type E1A in terms of prolongation of animal survival time (**Figure 11**). Results obtained from studies on a systemic delivery of wild type E1A in combination with or without chemotherapy (paclitaxel) have been published recently in the journal of ***Cancer Gene Therapy*** (2004, 11:594-602, also see appendix).

In order to understand the molecular mechanisms underlying E1A-mediated tumor- and metastasis-suppression, we recently discovered that expression of E1A also repressed the expression of a chemokine receptor CXCR4 expression under both normoxia and hypoxia conditions and blocked its response to SDF-1α, a ligand for CXCR4, which contributed partly to E1A-mediated repression of cell invasion and migration *in vitro* and tumor metastasis *in vivo* (**Figure 12-13**). A manuscript summarizing these results is in preparation and will be sent to the journal of ***Cancer Research*** as **Advances in Brief**.

Although we are not at the time to construct an alternative E1A construct for gene therapy based on the above results, we have constructed a C-terminal E1A mutant and tested its *in vivo* effect on tumor growth in animal models. Since we have not been able to establish a stable cell lines with C-terminal expression, we have constructed a tet-on/off regulated wild–type E1A and C-terminal E1A inducible expression constructs and established tet-off/on inducible cell lines with wild–type E1A or C-terminal E1A expression in breast cancer MCF-7 cells. The biological effects of these stable cells and the therapeutic efficacy of this C-terminal mutant in comparison with wild-type E1A and a N-terminal mutant E1A gene in a gene therapy setting await further analysis.

To gain a global view of genes and proteins regulated by E1A, we also used cDNA microarray and proteomic technologies including 2-D gel electrophoresis and Ciphergen protein chip array to identify the target genes and proteins associated with E1A in breast cancer cells. Differentially expressed genes were found in E1A stable cells versus parental cells by either the cDNA microarray or 2-dimensional gel electrophoresis and Ciphergen protein chip technology (**Figure 14 A-B**). Althoguh these experiments are not directly related to the original proposal, they are relevant to E1A's proapoptotic effect and anti-tumor activity and to breast cancer. Therefore, I will continue to pursue on finding E1A associated molecules and also plan to wire the panoramic signaling networks associated with E1A

by systems biology approaches utilizing the genomic and proteomic technologies in an inducible E1A expression model system.

In addition to the work on the proposed experiment, in corroboration with my colleagues, we uncovered additional two new Akt target genes that associated with drug resistance: the first one is a universal growth inhibitor p21Cip1/WAF1. We found that Akt directly associated with, phosphorylated, and inactivated p21Cip1/WAF1, which correlated with oncogene *Her-2/neu*-mediated drug resistance and cell growth. This work was published in ***Nature Cell Biology*** (4) (see appendix). In addition, based on our 2-D peptide mapping results and the carboxy-terminal structure of p21Cip1/WAF1, a small peptide was designed, for therapeutic intervention purpose, to compete with endogenous p21Cip1/WAF1 for Akt binding and phosphorylation. Preliminary results showed that it partially reversed sensitivity to drugs of *Her-2/neu* overexpressing cells. Detailed study of this peptide's efficacy and mechanism in drug-sensitization is under way. Using a similar phospho-peptide, we generated anti-phospho p21Cip1/WAF1 polyclonal antibodies and evaluated their status in human cancer and its correlation with patients' survival (a paper summarizing these findings has been published in ***Clinical Cancer Research***, 2004; 10(11): 3815-24, also see appendix). Meanwhile, our further investigation on Akt activation and drug resistance leads to the discovery of another Akt target gene, p53, a king tumor suppressor and also a key pro-apoptotic gene, which is inactivated by Akt in breast cancer cells. This finding was also published in ***Nature Cell Biology*** (5) (see appendix). Further investigations on additional Akt target genes lead us to identify a critical oncogenic peptidyl prolyl isomerase Pin1 that also associates with Akt and maintains Akt activity and stability (**Figure 15-16**). A manuscript summarizing these findings is in preparation and will be submitted as a letter to ***Nature***.

Key research accomplishments:

(1). We mapped domains of E1A responsible for downregulation of VEGF expression.

(2). Evaluated the chemosensitization effect of E1A by systemic E1A gene therapy approach. (Cancer Gene Therapy, 2004).

(3). We have found that upregulation of p38 activity and downregulation of Akt activity are necessary and sufficient for E1A–mediated sensitization to apoptosis induced by serum–starvation, TNF-α, UV-irradiation, and different categories of anticancer drugs (MCB, 2003).

(4). We have found that E1A downregulated a protein phosphatase PPM1D expression, which is amplified/overexpressed in human breast cancer.

(5). We identified that a few novel proteins associated with E1A by antibody array approaches and some of them have been also confirmed by co-IP and western blot analysis.

(6). We have found that upregulation of PP2A activity by E1A contributes to E1A-mediated downregulation of Akt activation and correlates with E1A-mediated chemosensitization (Cancer Research, 2004).

(7). We provided data to support that repression of VEGF expression by E1A might be achieved by inhibition of the HAT activity of p300 through binding with pRB and HDAC1 by antibody array and Co-IP approaches.

(8). We have mapped that CR2 domain of E1A is required for E1A-mediated downregulation of Akt, upregulation of PP2A/C and p38 (MCB, 2003; Cancer Research, 2004).

(9). We have found that E1A-mediated downregulation of a chemokine receptor CXCR4 contributed in part to E1A-mediated inhibition of cell invasion and migration in vitro and tumor metastasis in vivo (manuscript in preparation).

(10). We have identified a novel mechanism that contributed to maintain Akt activation in human cancer via a peptidyl prolyl insomerase Pin1 mediated conformation change (manuscript in preparation).

Reportable outcomes:

1. Research paper published:

- **Liao Y**, Hung MC: A new role of protein phosphotase 2A in adenoviral E1A protein mediated sensitization to anti-cancer drug–induced apoptosis in human breast cancer cells. ***Cancer Res*** September 1, 2004; 64: (17): 5938-42.
- Xia WY, Chen JS, Zhou X, Sun PR, Lee DF, **Liao Y**, Zhou BH, Hung MC: Phosphorylation/cytoplasmic localization of p21 CIP1/WAF1 is associated with HER2/neu overexpression and provides a novel combination predictor for poor prognosis in breast cancer patients. ***Clin. Cancer Res***. 2004; 10(11): 3815-24.
- **Liao Y**, Zou YY, Xia WY, and Hung MC**:** Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by a non-viral mediated systemic delivery of E1A gene in orthotopic xenograft human breast cancer. ***Cancer Gene Therapy***, 2004; 11(9): 594-602. .
- **Liao Y**, Hung MC: "The regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenovrial protein E1A-mediated sensitization to apoptosis." ***Molecular & Cellular Biology***, 2003; 23(19): 6836-6848, 2003.

- **Liao Y**, Zou YY, Xia WY, Hung MC: Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer. ***Mol. Therapy*** 7 (5): 1074 Part 2, 2003
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- **Liao Y***, Zhou BP*, Xia WY, Zou YY, Spohn B, Hung MC: HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. ***Nature Cell Biol.*** 2001; 3(11): 972-982 (*equal contribution)
- **Liao Y,** Zou YY, Xia WY, and Hung MC**:** Chemosensitization by adenovirus E1A through modulation of apoptotic signalings. ***Clin. Cancer Res.*** 2001; 7(11):3777s (suppl.)
- Zhou BP, **Liao Y**, Xia WY, Spohn B, Lee MH, Hung MC: Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. ***Nature Cell Biol.*** 2001; 3(3): 245-252

Book Chapter:

Liao Y, Hung MC: **Novel Approaches for Chemosensitization of Breast Cancer Cells.** In: Mechanism of Breast Cancer Chemoresistance (First Edition), Ed: Dihua Yu and Mien-Chie Hung, Landes Bioscience, Georgetown, TX 78626, 2005

Liao Y: Antisense Therapeutics and Application in Liver Cancer. In: Advances in Basic and Clinical Aspects of Liver Cancer Metastasis and Treatment (First Edition), Ed: Tang ZY, Shanghai Science & Technology Press, Shanghai, 2003. P:205-248 (in Chinese)

2. Abstracts and presentations:

- **Liao Y**, Hung MC: A new role of protein phosphotase 2A in adenoviral E1A protein mediated sensitization to anti-cancer drug–induced apoptosis in human breast cancer cells. International Conference on Tumor Progression and Therapeutic Resistance, Philadelphia, PA, November 8-9, 2004
- Li YM, Cheng X, Pan Y, Xie X, **Liao Y**, Yu D, Hung MC: Adenovirus E1A inhibits tumor metastasis through repressing chemokine receptor CXCR4 expression. Poster to be presented to the 96th AACR Annual Meeting, Anaheim, CA, April 16-20, 2005, and manuscript to be submitted to ***Cancer Res***.
- **Liao Y** and Hung MC: E1A-mediated sensitization to anti-cancer drug-induced apoptosis in human breast cancer. The 24th Congress of the International Association for Breast Cancer Research, Sacramento, California, November 1-5, 2003
- **Liao Y**, Zou YY, Xia WY, and Hung MC: Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in orthotopic xenograft human breast cancer. Proceedings of the 6th Annual Meeting of the American Society of Gene Therapy, Washington, DC., June 2-8, 2003
- **Liao Y,** Zou YY, Xia WY, and Hung MC: Chemosensitization through adenovirus E1A-mediated signal integration. Proceedings of the Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 25-28, 2002
- Zhou BP, **Liao Y**, Xia WY, Zou YY, Sphon B, and Hung MC: Her-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Proceedings of the Ninety-Second Annual Meeting of the American Association for Cancer Research,* 43: 818, 2002

- **Liao Y**, Zou YY, Xia WY, Hung MC: Chemosensitization by adenovirus E1A through modulation of apoptotic signalings. Molecular Targets and Cancer Therapeutics, AACR-NCI-EORTC Annual Meeting, Miami Beach, FL, Oct.29-Nov. 2, 2001
- **Liao Y**, Zou YY, Xia WY, Lee WP, Hung MC: Modulation of apoptotic threshold: enhanced anti-tumor therapy through downregulating Akt and upregulating p38 by adenovirus E1A in human breast cancer cells. Proceedings of the 92nd AACR Annulal Meeting, New Orleans, LA, March 24-28, 2001
- **Liao Y**, Spohn B, Lee WP, Zou YY, Hung MC: Functional domains of adenovirus type 5 E1A in E1A tumor suppression and sensitization to Taxol-induced apoptosis. Proceedings of the 91st AACR Annulal Meeting, San Francisco, CA, April 1-5, 2000, 41: 351-352
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- Paul PW, **Liao Y**, Su Z, Spohn B, Ueno N, Lafoe D, Anklesaria P, Hung MC: In vivo tumor inhibitory activity of the C-terminal fragment of the adenovirus 5 E1A protein. Proceedings of the 2nd Annual Meeting of American Society of Gene Therapy, Washington, DC, June 9-13, 1999, P: 176a.

3. Stable cell lines and constructs:

   Stable Wip1 promoter driving luciferase reporter expression construct in MCf-7 cells

   pSuper-Wip1 siRNA expression construct in MCF-7 cells

   Serial deletion mutation constructs of Wip1 and Akt

   Serial point mutation constructs of Akt

Conclusion:

These studies on the molecular mechanisms underlying E1A's tumor suppression and chemosensitization can help us to better design an alternative E1A construct for future gene therapy. Specifically, identification of additional E1A target genes, such as PPM1D, PP2A, Akt and p38, may help us in finding novel ways to treat cancer patients by targeting the deregulated signals. The combination of E1A gene therapy with Taxol or other chemotherapeutic drugs is one potential new therapeutic approach for the treatment of cancer patients, as we have shown in the animal models. The mutant E1A construct(s), if it works in animal model, could potentially be translated into the clinic and be of a great benefit to breast cancer patients.

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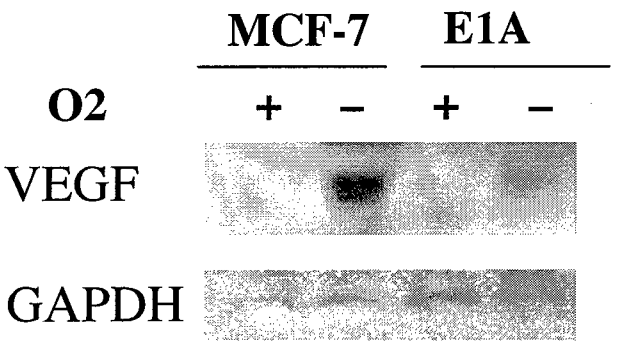
Appendices:

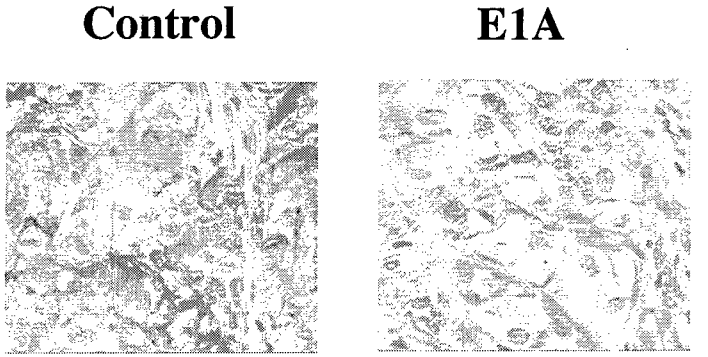
Reprints (NCB, x2, MCB, x2, Cancer Res., Cancer Gene Therapy, Clin. Cancer Res., Oncogene)
Book chapter
Grant proposal and award notice (Susan G. Komen Foundation)
CV (updated to June, 05)
Personal Information (Address, telephone number, Fax, Email)

Figure 1. **E1A downregulate VEGF expression *in vitro* (A) and *in vivo (B-C)*.**

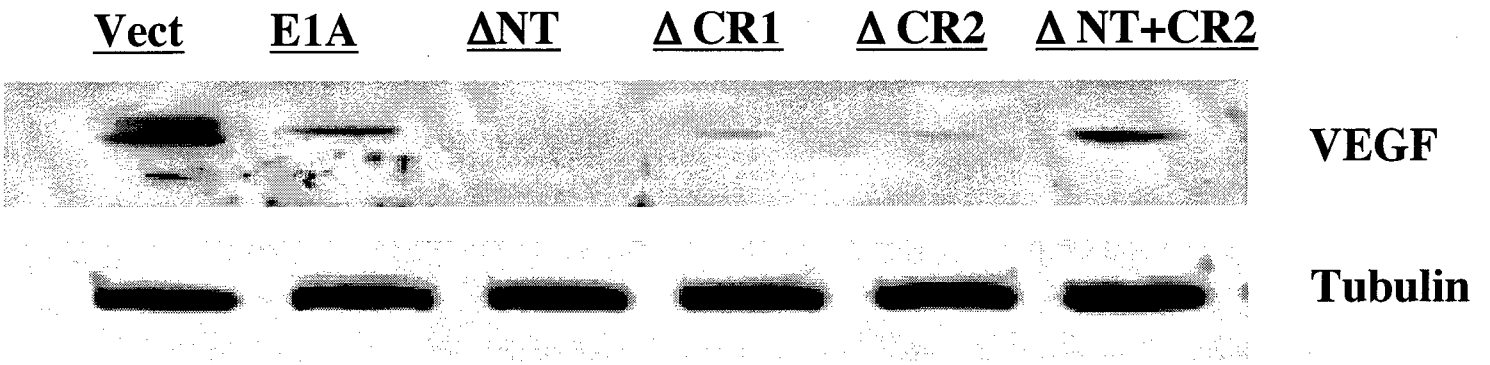
A. Northern Blot analysis of VEGF expression in cell culture *in vitro*.



B. Immunohistochemistry analysis of VEGF expression in tumor tissue *in vivo*.



C. Western blot analysis of VEGF expression in tumor tissues *in vivo* obtained from inoculation of vector control cells or wild-type E1A and different domain mutant E1A stable cells.



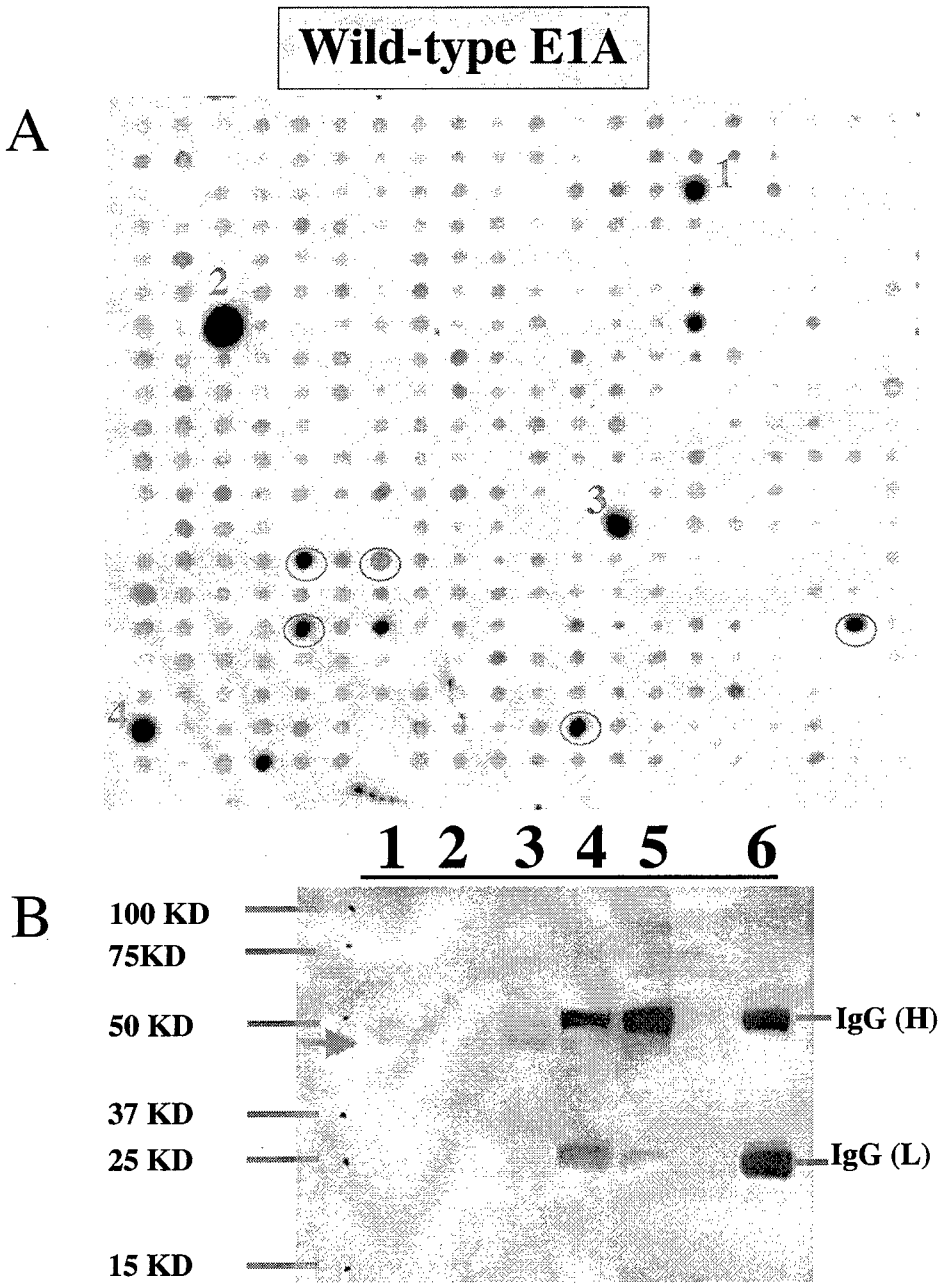
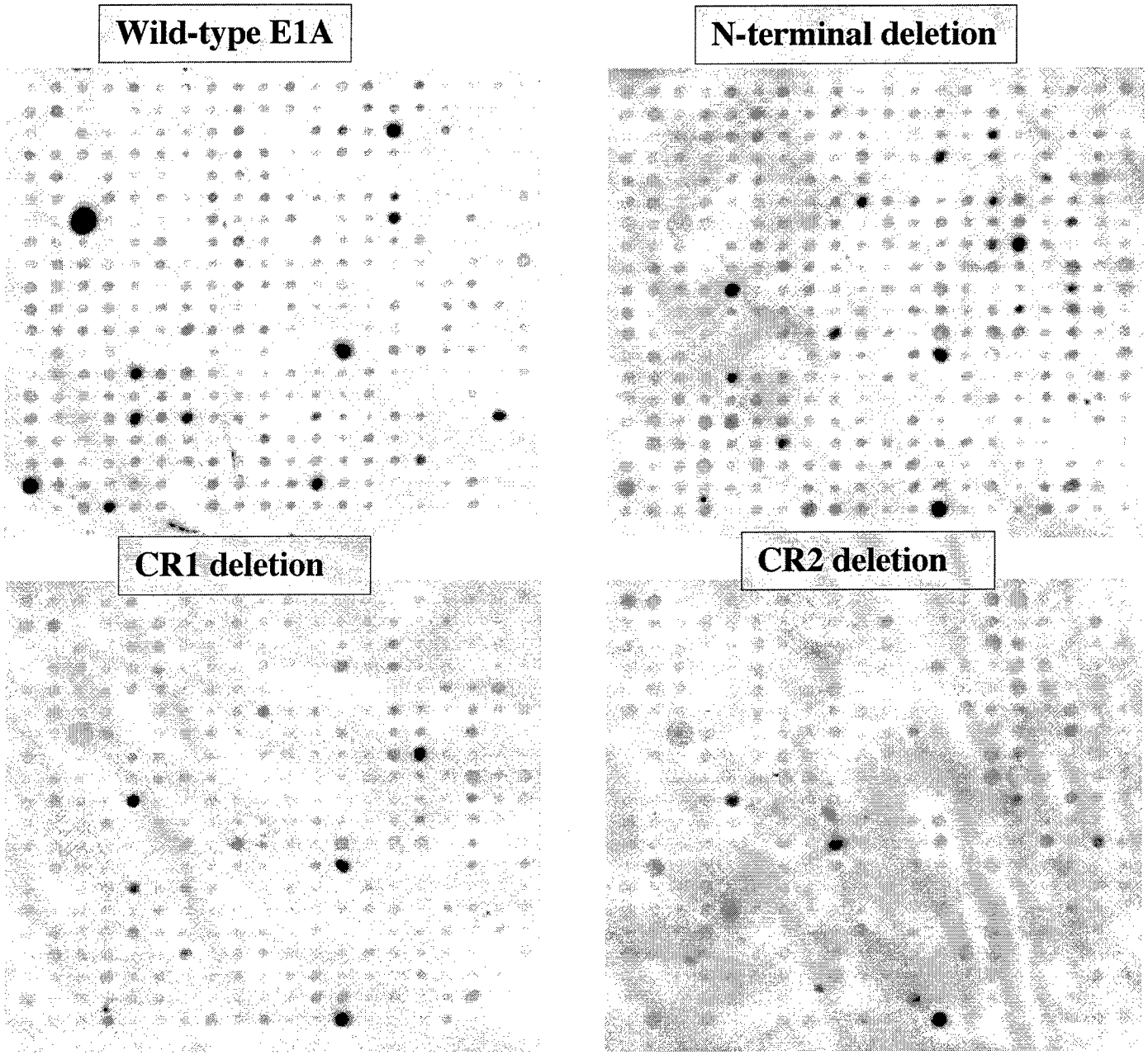


Figure 2

A. Antibody array of proteins associated with E1A. Number 1, 2, 3, and 4 represent proteins that have not been reported to be associated with E1A, but were identify by antibody array and their association with E1A were further supported by co-immuoprecipitation as shown in Figure B. Circled proteins are those previously reported to be associated with E1A.

B. Proteins marked as 1 to 4 in Figure A were immunoprecipitated by respective antibodies and bloted against anti-E1A antibody (M73). Number 5 represents a positive control by using anti-E1A antibody for the immunoprecipitation. Number 6 represents a negative control by using normal mouse IgG. Arrow indicates the E1A protein band.

C


Wild-type E1A
N-terminal deletion
CR1 deletion
CR2 deletion

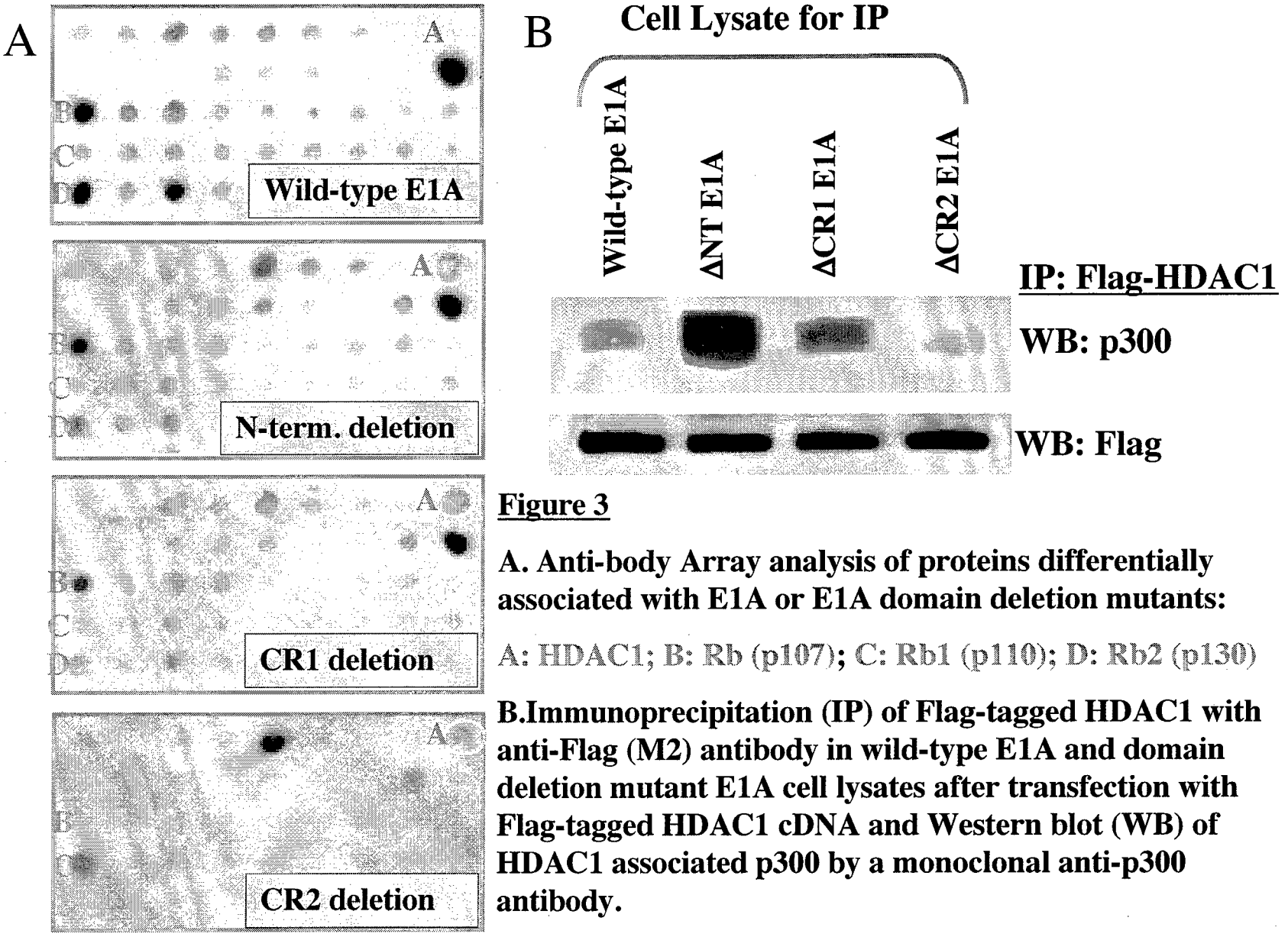
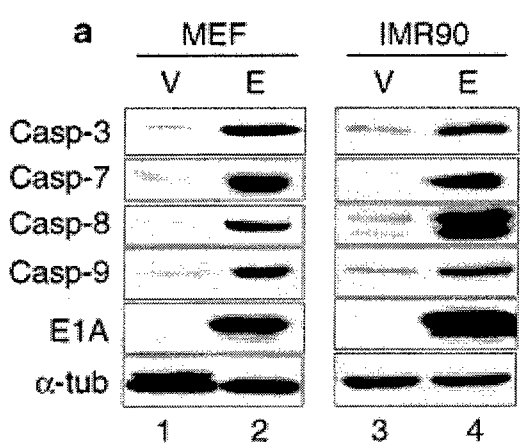


Figure 3

A. Anti-body Array analysis of proteins differentially associated with E1A or E1A domain deletion mutants:

A: HDAC1; B: Rb (p107); C: Rb1 (p110); D: Rb2 (p130)

B.Immunoprecipitation (IP) of Flag-tagged HDAC1 with anti-Flag (M2) antibody in wild-type E1A and domain deletion mutant E1A cell lysates after transfection with Flag-tagged HDAC1 cDNA and Western blot (WB) of HDAC1 associated p300 by a monoclonal anti-p300 antibody.



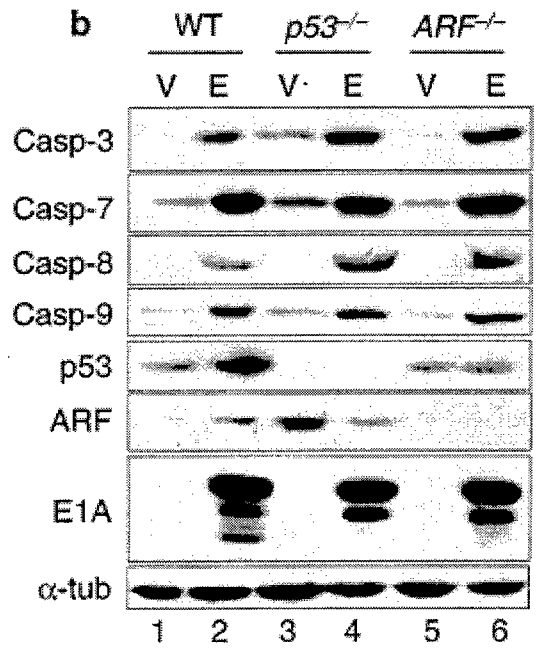
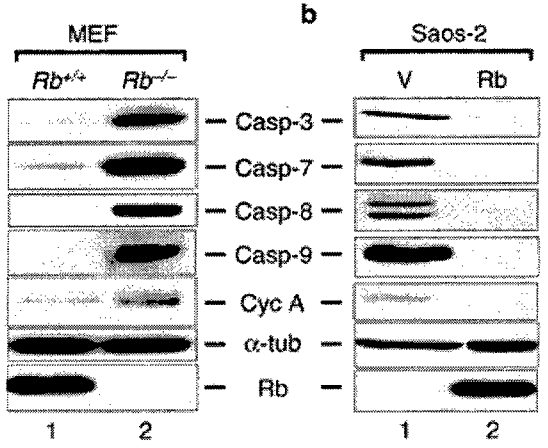
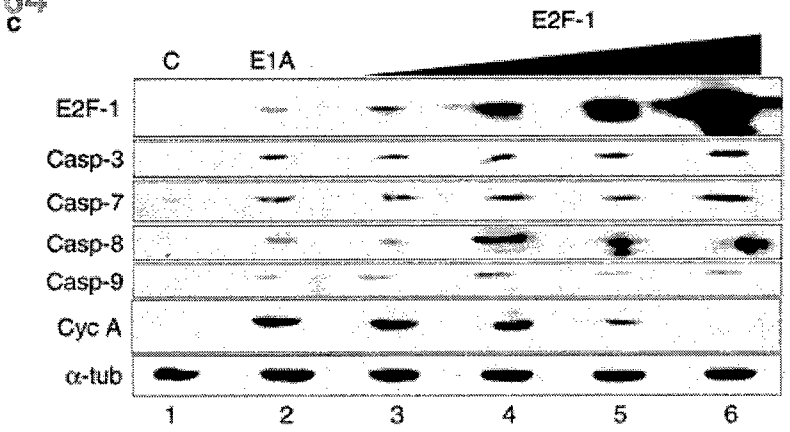
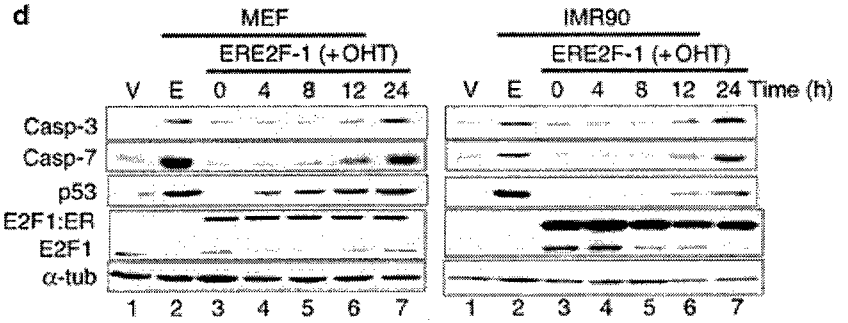


Figure 4, adopted from Nahle Z, et al

Nature Cell Biology
november
2002 volume 4
issue 11
pp 859 - 864







A

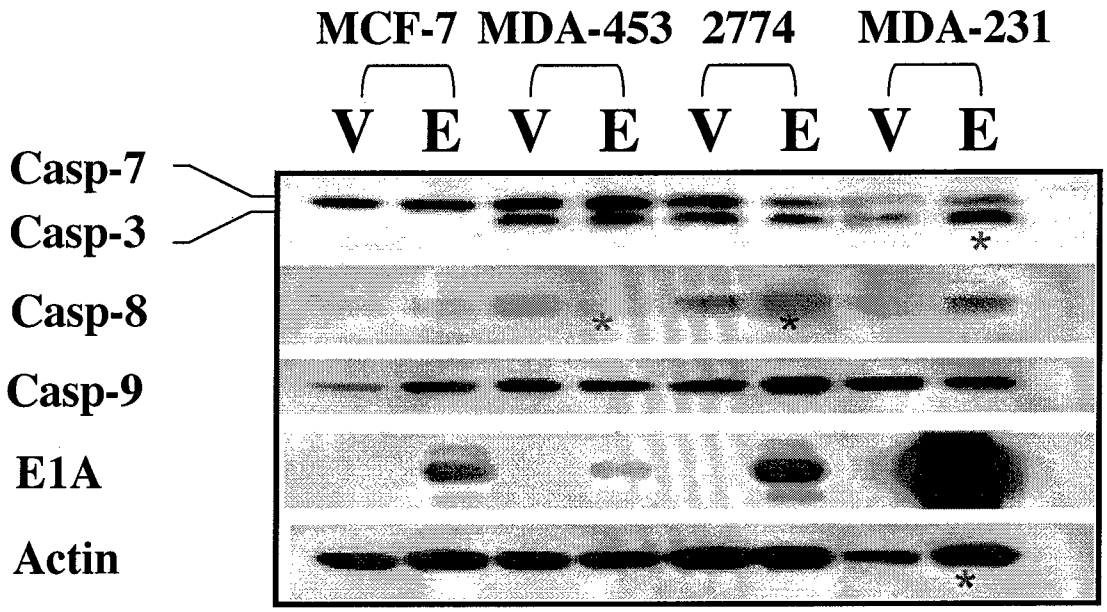


Figure 5

A. Expression of E1A in human carcinoma cells may not results in transcriptional upregulation of caspase proenzymes

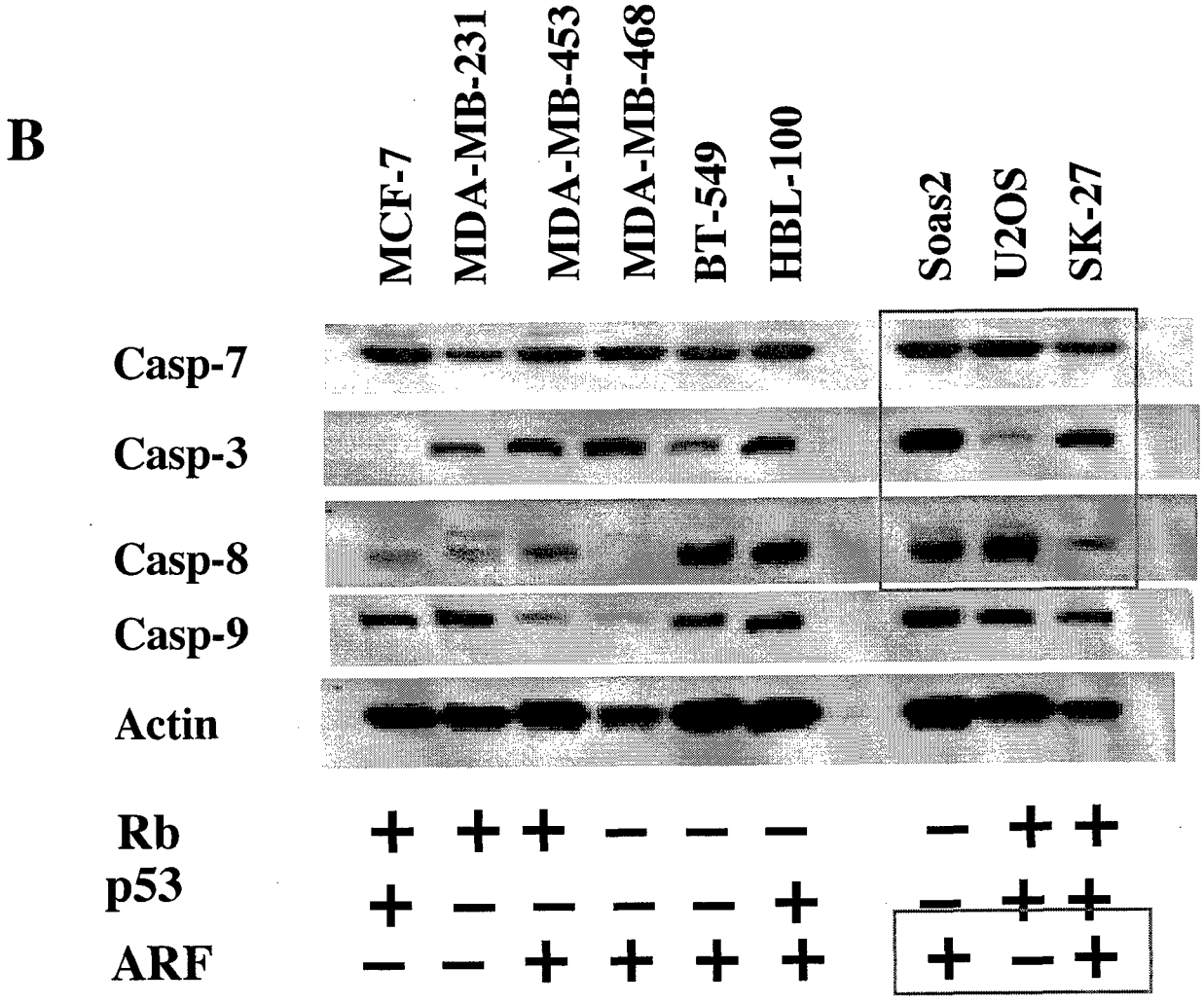


Figure 5

B. Neither p53 nor Rb status affected caspase proenzyme expression in human cancer cells

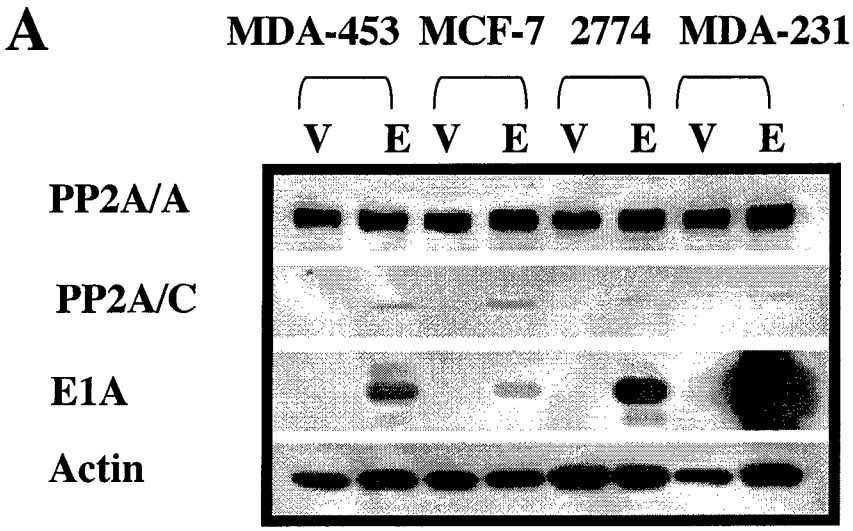


Figure 6.

A. E1A upregulates the catalytic, but not the regulatory subunit of PP2A

B. E1A upregulates PP2A phophatase activity through upregulation of PP2A/C, the catalytic subunit of PP2A.

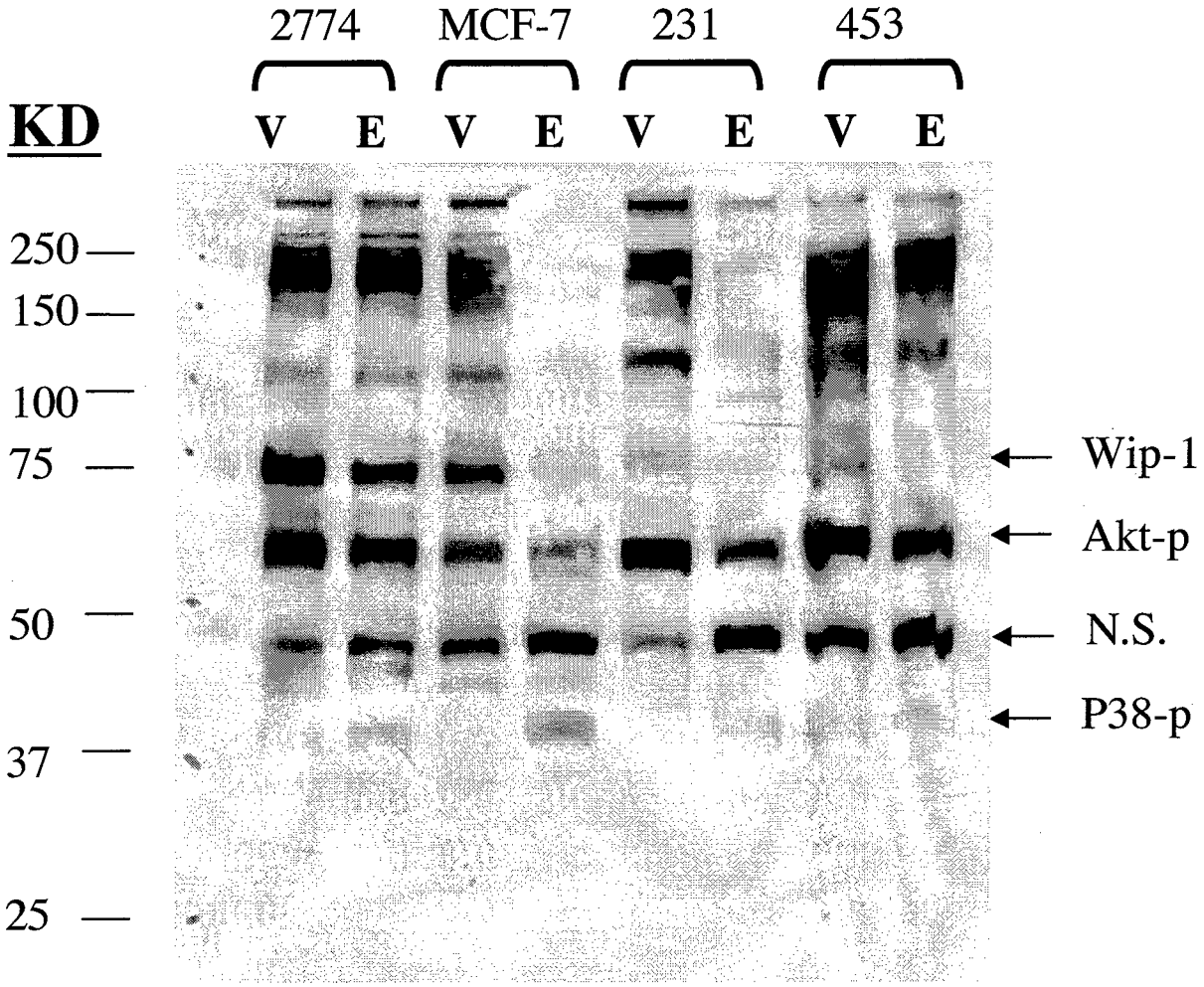
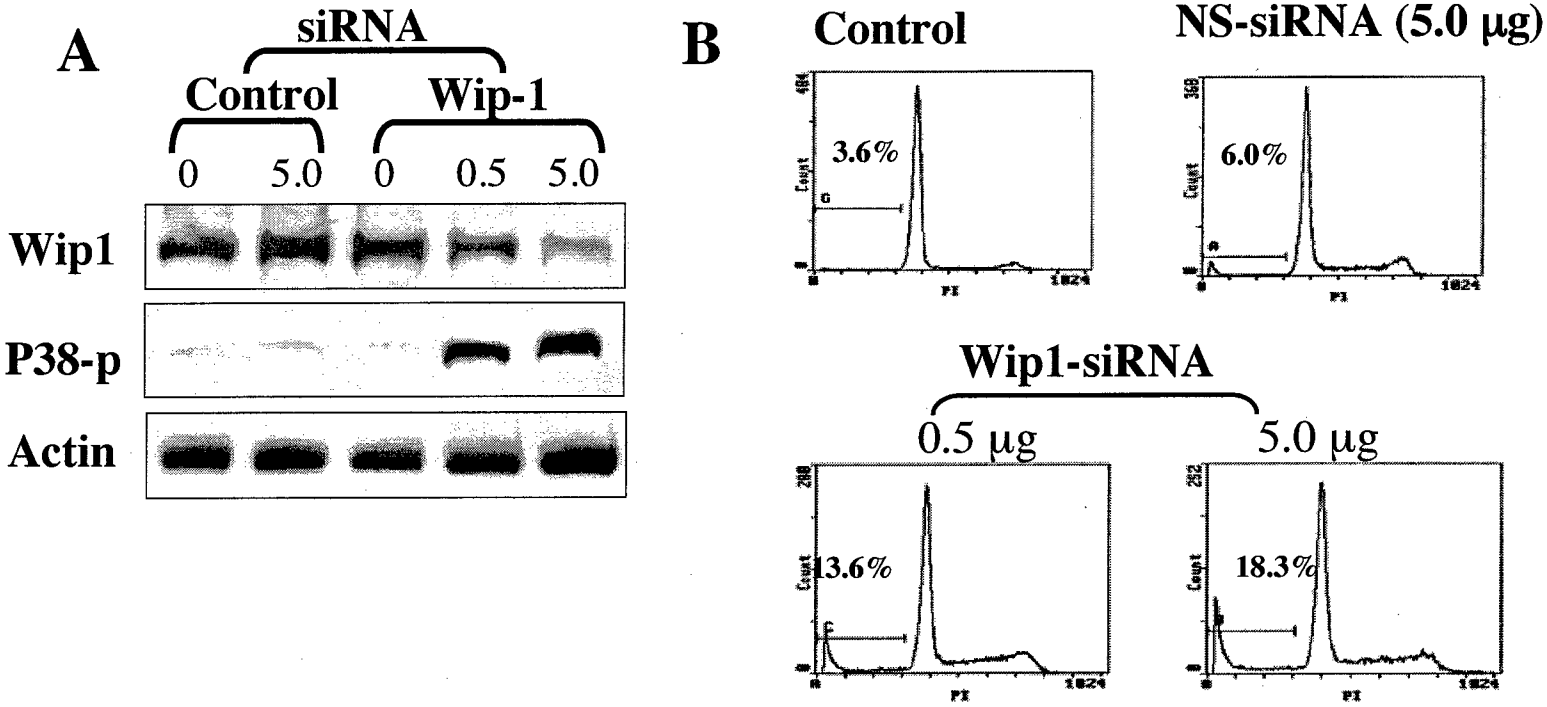


Figure 7 Repression a protein phosphatase PPM1D (Wip-1) expression by E1A in stable E1A expression cells correlates with its effect on Akt and p38 kinase phosphorylation in breast cancer 2774, MCF-7, MDA-MB-231, and MDA-MB-453.

Figure. 8 **Blockage of Wip1 expression by specific siRNA against Wip1 results in enhancement of p38 phosphorylation (A) and spontaneous apoptosis (B) in breast cancer cell line MCF-7 cells.**



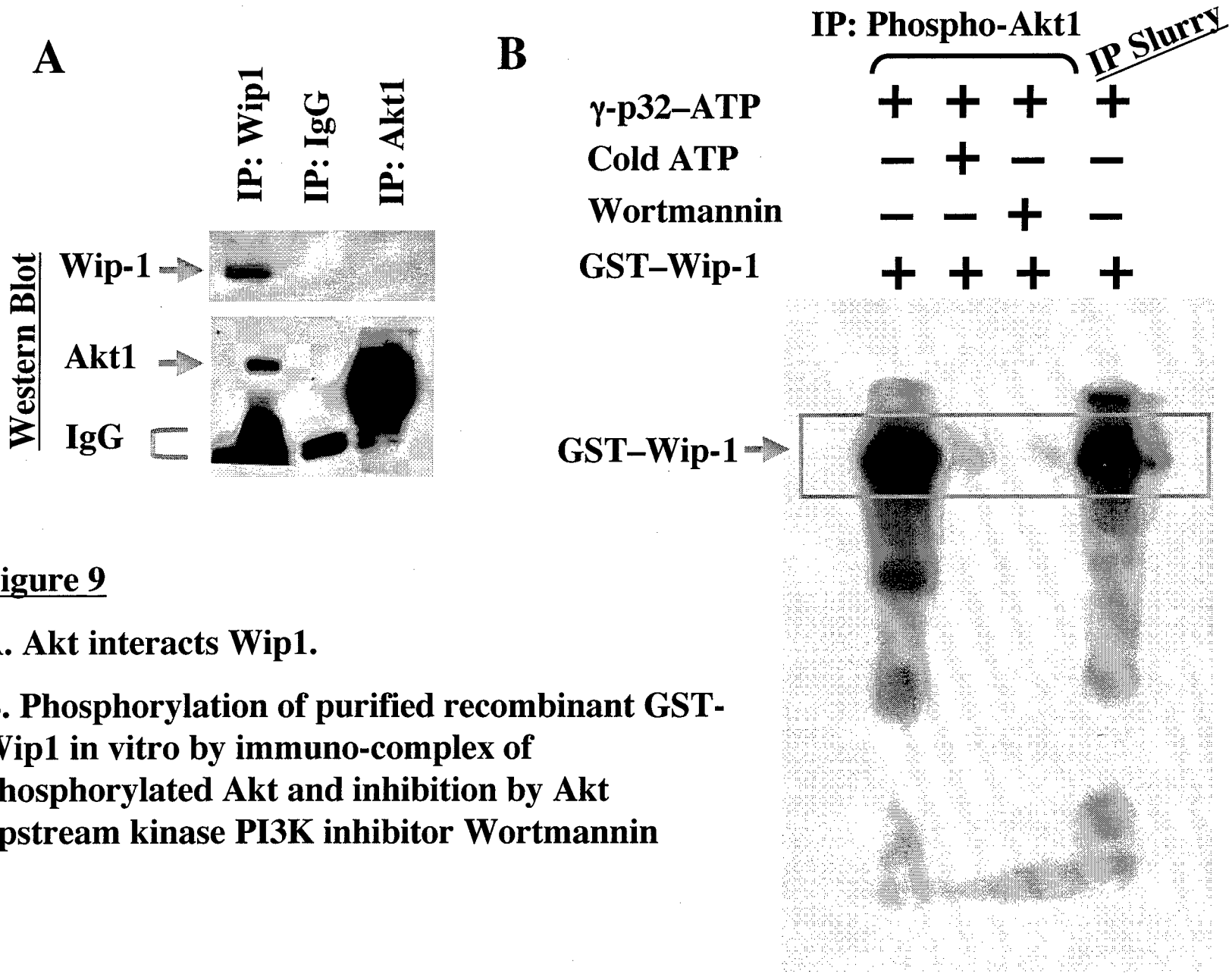


Figure 9

A. Akt interacts Wip1.

B. Phosphorylation of purified recombinant GST-Wip1 in vitro by immuno-complex of phosphorylated Akt and inhibition by Akt upstream kinase PI3K inhibitor Wortmannin

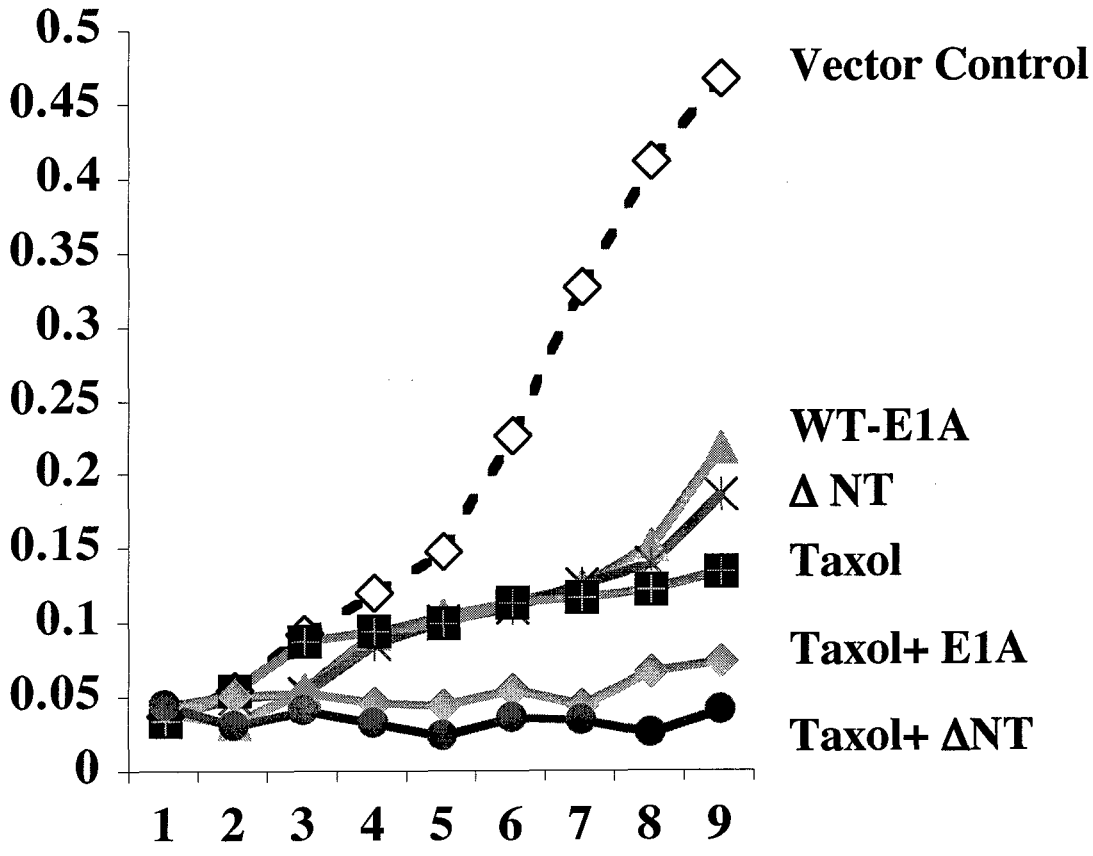


Figure 10. Systemic gene therapy effect of wild-type E1A or N-terminal deletion mutant E1A (Δ NT) with or without combination with paclitaxel (Taxol) chemotherapy in breast cancer xenograft established by inoculation with MDA-MB-231 cells in nude mice.

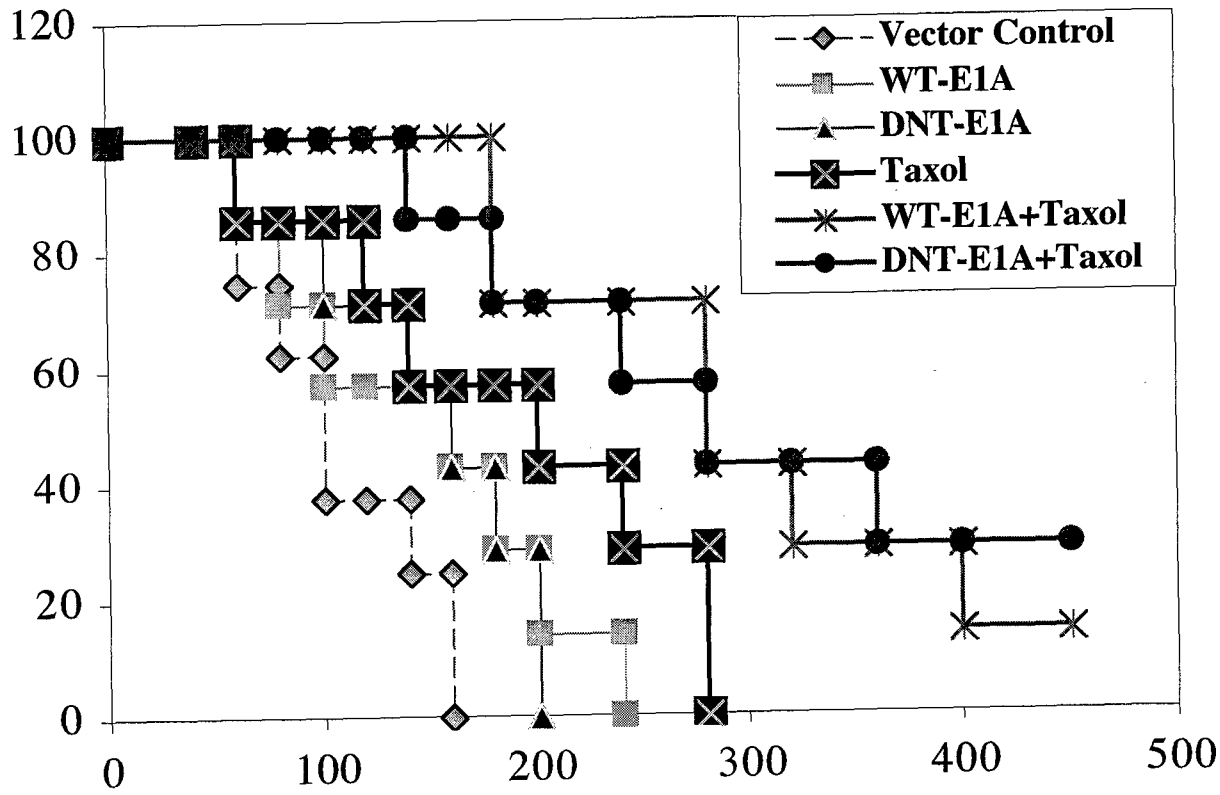
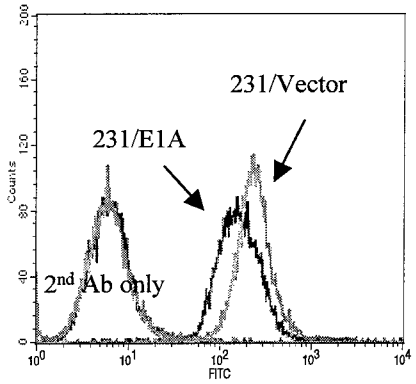


Figure 11. Survival rate of Systemic gene therapy effect of wild-type E1A (WT-E1A) versus N-terminal deletion mutant E1A (DNT-E1A) with or without combination with paclitaxel (Taxol) chemotherapy in breast cancer xenograft established by inoculation with MDA-MB-231 cells in nude mice.

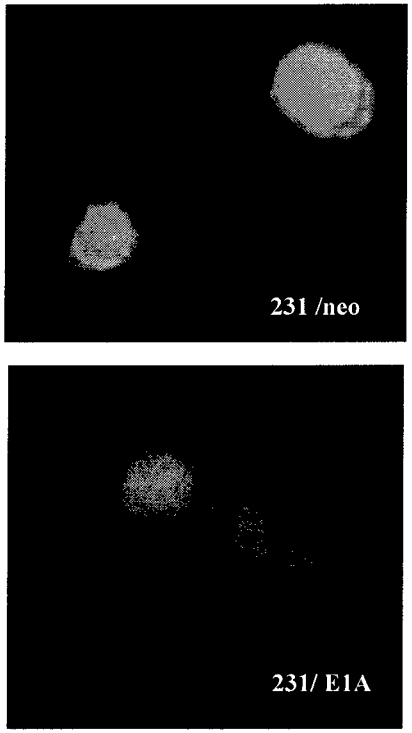
Figure 12. E1a represses the chemokine receptor CXCR4 expression.

Malignant cancer cell lines and its E1a-stable transfectants were stained for the CXCR4 Antibody (MAB 172, R&D) and analyzed using FACS **(A),** immunofluorescent staining **(B),** Western blot **(C),** and Northern blot **(D).** 231/neo and 231/E1a cells were stained with anti-CXCR4 (green) antibody and nuclear dye DAPI (red) and examined under a fluorescent microscope (Zeiss).

A



B



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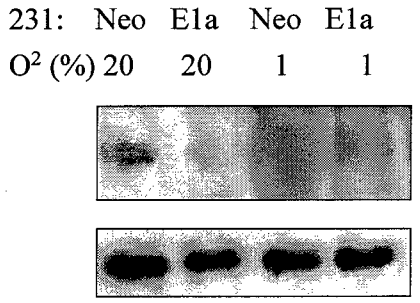

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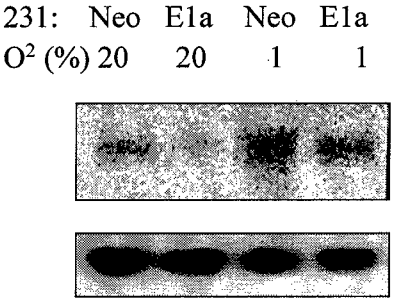


Figure 13. Repression of CXCR4 expression by E1A contributed to E1a-mediated inhibition of cell invasion and migration *in vitro*. (**A**) Invasive assays were performed using the Boyden Chamber assay coated with matrigel, which mimics basement membrane composition, and SDF-1α, a ligand for CXCR4, as a chemoattractive agent in the lower chamber. For the blocking assay, either CXCR4 blocking antibody (1-10 μg/ml) was added into cell culture. Cells were counted in triplicate wells and in five identical experiments. (**B**) Quantification of invasion assays. *, $P < 0.05$. (**C**) The migration assay was performed in a manner similar to the invasive assay, except in the absence of matrigel. Quantification of migration is shown. *, $P < 0.05$.

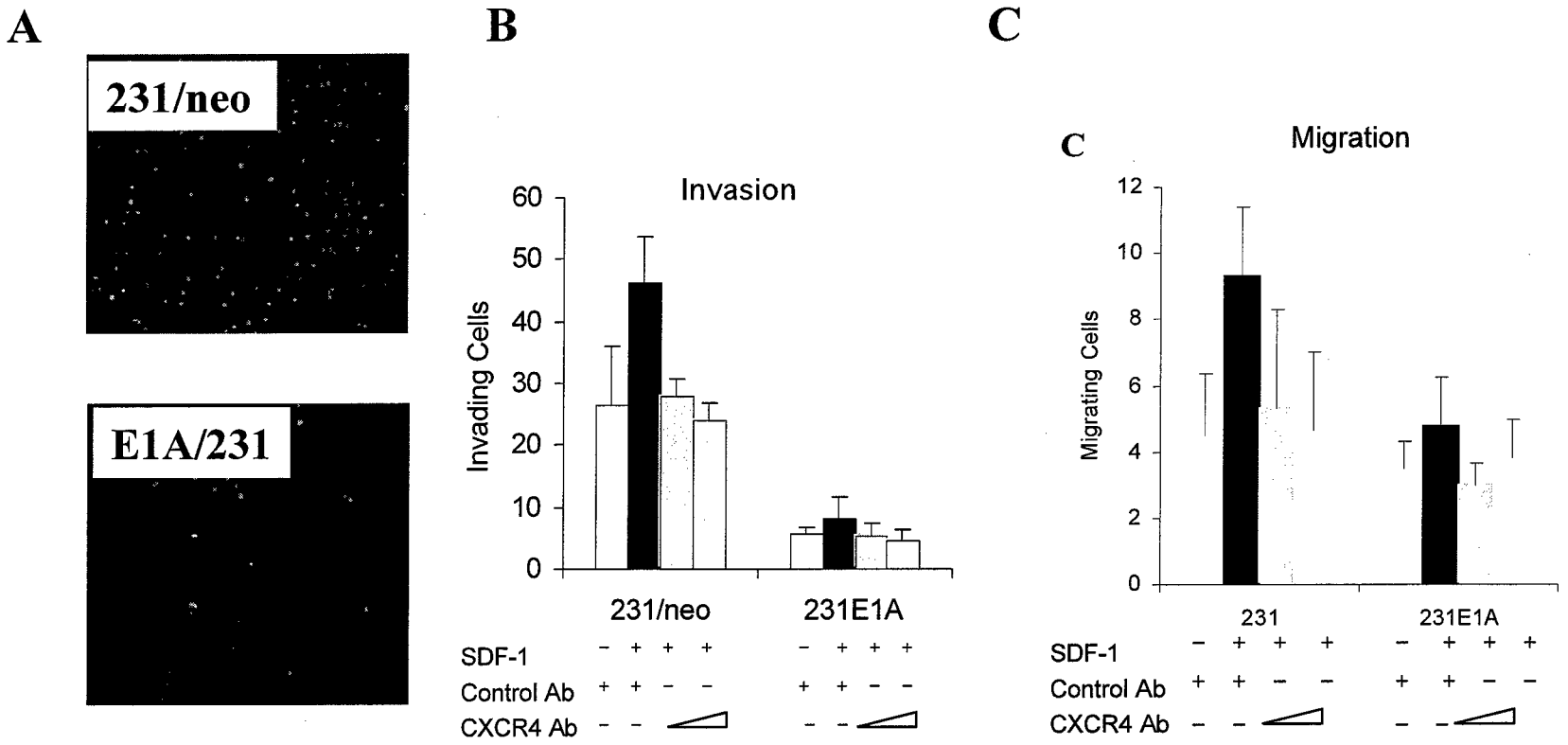
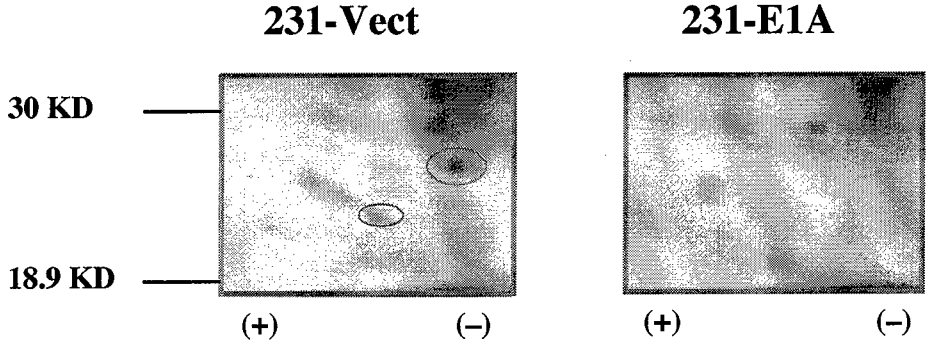


Figure 14

Proteins differentially expressed in 231-E1A stable cells identified by using 2D gel (A) and Ciphergen protein chip profiling analysis (B)

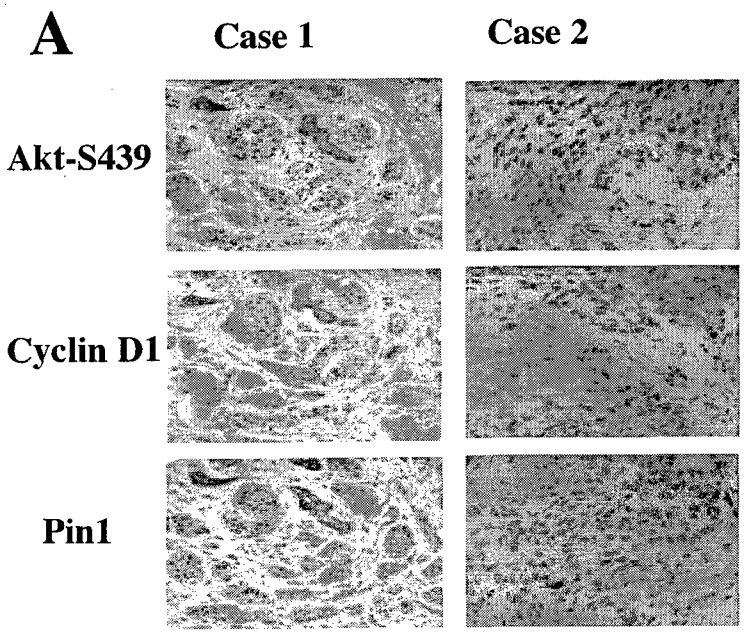
A. E1A down-regulated proteins identified by 2D gel electrophoresis.

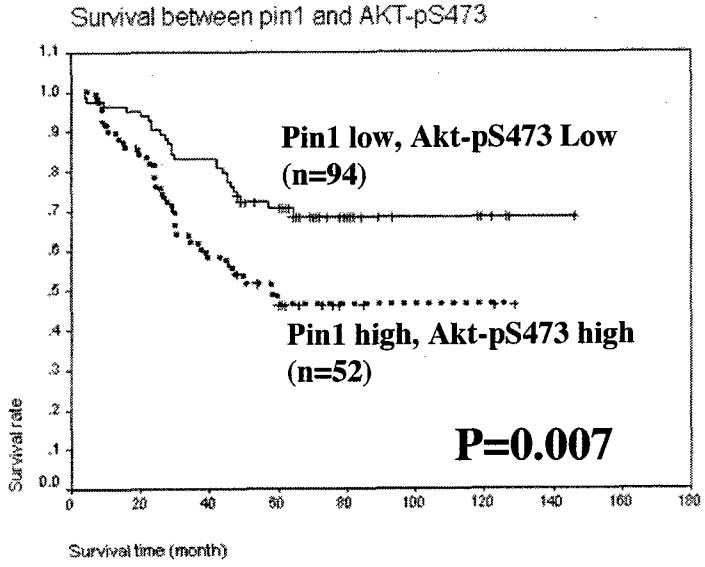


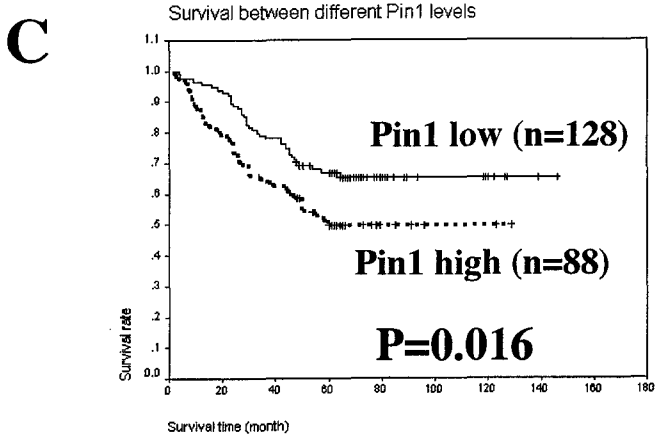
B. Differentially expressed proteins in 231-E1A cells identified by Ciphergen proteinchip analysis

Type of Protein chip	Up-regulated proteins (KD)	Down-regulated proteins (KD)
H4	**37.5, 43.7, 48.8, 53.1, 87.4, 4.36, 6.81**	**45.4, 56.5, 65.7, 69.7, 6.25**
SAX2	**34.9, 61.1, 7.9**	**12.4, 17.5, 25.9, 148.8, 6.18**
WCX2	**37.4, 3.3**	**69.7**
IMAC-cu	**37.3, 40.6**	**49.6, 65.7**

Figure 15. Expression of Pin1 correlates with Akt phosphorylation at S473 and contribute to a poor survival rate in human breast cancer. (A). Immunohistochemistry of Pin1, Akt S473, and Cyclin D1 expression in breast tumor; (B)-(D). Patient survival rate.







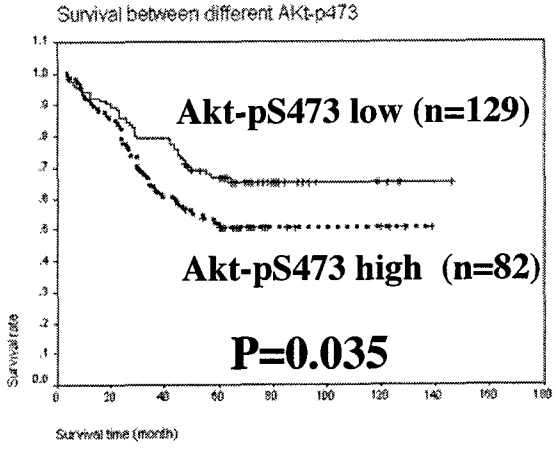
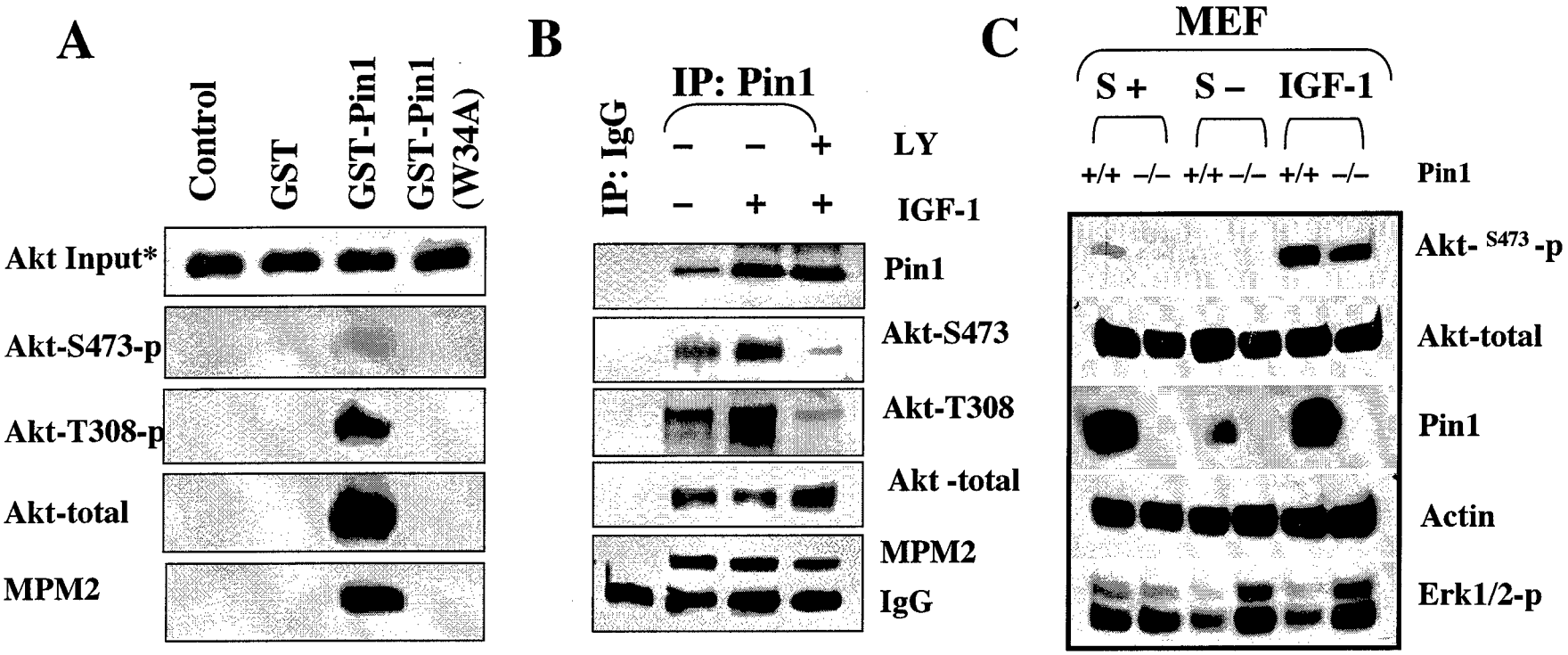


Figure 16. Pin1 interacts with Akt, which is independent of Akt phosphorylation at neither T308 nor S473, while knockout Pin1 results in reduced basal and IGF-1-stimulated Akt phosphorylation at S473.

(**A**). GST-Pin1 can pull down Akt but the Pin1 mutant (W34A) which lost the ability to bind with substrate also lost the ability to bind with Akt; (**B**). Pin1 binding with Akt is independent of Akt phosphorylation at neither T308 nor S473; (**C**). In Pin1 knockout mouse embryo fibroblast (MEF) cells, Akt phosphorylation at S473 in the presence (S+) or absence (S–) of serum or IGF-1 stimulation was compromised. This result suggests that Pin1 is required for maintaining Akt phosphorylation.



RESUME

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- **Liao Y,** Hung MC: Integration of the pro- and anti-apoptotic signals by protein phosphatase 2a in adenoviral E1A-mediated chemosensitization. Proceedings of the Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA, June 8-12, 2005
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CHAPTER 12

Novel Approaches for Chemosensitization of Breast Cancer Cells:
The E1A Story

Yong Liao, Dihua Yu and Mien-Chie Hung*

Abstract

The adenoviral E1A-mediated sensitization to a variety of anti-cancer drug-induced apoptosis is well-established phenomenon on different types of cell systems. However, the mechanisms underlying E1A-mediated chemosensitization are still not fully understood. Recent studies demonstrate that E1A-mediated sensitization to drug-induced apoptosis can occur via multiple pathways; some of which depend on the expression of functional p53 and/or p19ARF proteins, while some are not. In human breast cancer cells with Her-2/neu overexpression, which usually are more resistance to anti-cancer drugs than cells without Her-2/neu overexpression, may be sensitized through E1A–mediated downregulation of Her-2/neu. Alternatively, E1A can induce sensitization to anticancer drugs in cancer cells or normal diploid fibroblast cells through upregulating the expression of caspase proenzymes, or downregulating the activity of a critical survival factor Akt and/or upregulating the activities of a pro-apoptotic kinase p38 and a protein phosphatase PP2A, etc. This review summarizes these progresses and proposes a plausible feed-forward model for E1A-mediated chemosensitization in human breast cancer cells.

Introduction

Breast cancer is the most common malignancy in American and northwestern European women. In 2005, 211,240 new cases of invasive breast cancer will be diagnosed among American women, as well as an estimated 58,490 additional cases of in situ breast cancer, and 39,800 women are expected to die from this disease. Only lung cancer accounts for more cancer deaths in women. Men are also susceptible to the disease, with an estimated 1,690 cases and 460 deaths in 2005 in the United States.[1] Despite recent advances in the treatment of breast cancer, survival rates for patients with metastatic breast cancer remain poor. Chemotherapeutics are the most effective treatment for metastatic tumors. However, the ability of cancer cells to become simultaneously resistant to different drugs—a trait known as multidrug resistance—remains a significant impediment to successful chemotherapy. Three decades of multidrug-resistance research have identified a myriad of ways in which cancer cells can elude chemotherapy, and it has become apparent that resistance exists against every effective drug, even the newest agents.[2] Therefore, the ability to circumvent drug resistance is likely to improve the efficacy of chemotherapy.

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The adenoviral type-5 and type-2 early region 1A (E1A) proteins were reported originally as an oncogene that could cooperate with other viral and cellular oncogenes to transform primary culture cells but not established cell lines, as distinct from the type-12 E1A, a potent oncogene that can transform established cell lines.[3] However, E1A has not been associated with human malignancies despite extensive studies. Instead, E1A was shown to suppress experimental metastasis of rodent cells transformed by the ras oncogene[4,5,6] and the Her-2/neu oncogene[7-9] and metastasis of certain human cancer cell lines.[10,11] In addition, increasing experimental results indicate that E1A can inhibit the tumorigenicity of both the transformed rodent cells and the human cancer cell lines.[7,8,12] Therefore, based on its ability to suppress both tumorigenicity and metastasis, E1A has been considered as a tumor suppressor gene[3,11,13-20] and translated into multiple clinical trials.[3,21-27] In addition to tumor suppressor activities, expression of the E1A gene in stably transfected normal fibroblast and human cancer cells has also been shown to increase sensitivity to the in vitro cytotoxicity of several anticancer drugs (such as etoposide and cisplatin) in normal fibroblasts and sarcoma cells, doxorubincin in colon and hepatocellular carcinoma cells, gemcitabine in hepatocellular and breast cancer cells, and paclitaxel in breast and ovarian cancers.[28-39] In primary mouse embryo fibroblasts, E1A-mediated sensitization to apoptosis induced by ionizing radiation and chemotherapeutic drugs has been reported through a p53-dependent and $p19^{ARF}$-dependent pathway.[28,40-44] Moreover, a p53-independent mechanism for E1A-mediated chemosensitization to the anticancer drugs etoposide and cisplastin has been demonstrated in human cancer cell lines, including Saos-2 osteosarcoma cells.[30,31,42,45] So far, E1A has been shown to mediate sensitization to a wide variety of apoptotic stimuli, including serum starvation, ultraviolet (UV)- and γ-radiation, tumor necrosis factor (TNF)-α, and different categories of anticancer drugs. This review describes a framework for drug-induced apoptosis and summarizes some recent work that improves our understanding of the molecular mechanisms underlying the adenovirus E1A-mediated chemosensitization.

Mechansims of Apoptosis: Intrinsic Versus Extrinsic Apoptotic Pathways

Most chemotherapeutic drugs kill cancer cells by inducing apoptosis, and many similarities exist in cellular response to drug-induced apoptosis, regardless of their primary target.[46-52] Defects in apoptosis signaling contribute to resistance of tumors.[47,48,53,54] Apoptosis, from the Greek word for "falling off" or "dropping off" (as leaves from a tree), is defined by distinct morphological and biochemical changes mediated by a family of cysteine aspartic acid-specific proteases (caspases), which are expressed as inactive precursors or zymogens (pro-caspases) and are proteolytically processed to an active state following an apoptotic stimulus. To date, approximately 14 mammalian caspases have been identified and can be roughly divided into three functional groups: apoptosis initiator (including caspase-2, -9, -8, -10), apoptosis effector (including caspase-3, -6, -7), and cytokine maturation (including caspase-1, -4, -5, -11, -12, -13, -14).[55-57] Two separable pathways lead to caspase activation: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is initiated by ligation of transmembrane death receptors (Fas, TNF receptor, and TRAIL receptor) with their respective ligands (FasL, TNF, and TRAIL) to activate membrane-proximal caspases (caspase-8 and –10), which in turn cleave and activate effector caspases such as caspase-3 and –7. This pathway can be regulated by c-FLIP, which inhibits upstream initiator caspases, and inhibitor of apoptosis proteins (IAPs), which affect both initiator and effector caspases.[58-60] The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins, such as cytochrome *c*. Cytochrome *c*, released from the mitochondrial intermembrane space to cytoplasm, works together with the other two cytosolic protein factors, Apaf-1 (apoptoic protease activating factor-1) and procaspase-9, to promote the assembly of a caspase-activating complex termed the apoptosome, which in return induces activation of caspase-9 and thereby initiates the apoptotic caspase cascade.[56,57,61-63]

The primary regulatory step for mitochondrial-mediated caspase activation (the intrinsic pathway) might be at the level of cytochrome *c* release.[62,63] The known regulators of cytochrome *c* release are Bcl-2 family proteins.[62-65] According to their function in apoptosis, the mammalian Bcl-2 family can be divided into pro-apoptotic and anti-apoptotic members. The pro-apoptotic members include Bax, Bcl-Xs, Bak, Bok/Mtd, which contain 2 or 3 Bcl-2 homology (BH) regions, and molecules such as Bad, Bik/Nbk, Bid, Hrk/DP5, Bim/Bod, and Blk, which contains only the BH3 region. The anti-apoptotic Bcl-2 family members include Bcl-2, Bcl-XL, Bcl-w, A1/Bfl-1, Mcl-1, and Boo/Diva, which contain three or four regions with extensive amino acid sequence similarity to Bcl-2 (BH1-BH4).[65,66] Overexpression of the anti-apoptotic molecules such as Bcl-2 or Bcl-XL blocks cytochrome *c* release in response to a variety of apoptotic stimuli. On the contrary, the pro-apoptotic members of the Bcl-2 family proteins (such as Bax and Bid) promote cytochrome *c* release from the mitochondria. Pro-apoptotic and anti-apoptotic members of the Bcl-2 protein family can physically interact. For example, binding of BH-3 only proteins (e.g., Noxa, Puma, Bad, and Bim) to anti-apoptotic Bcl-2 proteins (e.g., Bcl-2 and Bcl-XL) results in activation of Bax and Bak.[64,65] In addition, there is considerable cross-talk between the extrinsic and intrinsic pathways. For example, caspase-8 can proteolytically activate Bid, which can then facilitate the release of cytochrome *c* and amplifies the apoptotic signal following death receptor activation.[52,56,57,61-63] Most of the anticancer agents either directly induce DNA damage or indirectly induce secondary stress-responsive signaling pathways to trigger apoptosis by activation of the intrinsic apoptotic pathway, and some can simultaneously activate the extrinsic receptor pathway. Therefore, molecules or signaling events that regulate the processes of apoptosis can also affect cellular response to drugs.

Factors and Key Molecules Involved in the Regulation of Apoptosis and Drug Response in Breast Cancer

Her-2/neu

The erbB2 gene (also known as HER-2, neu, and NGL) encodes a 185-kDa transmembrane glycoprotein (ErbB2) that belongs to the epithelial growth factor receptor (EGFR) family and heterodimerizes with other EGFR family members upon ligand stimulation. Similar to EGFR, the Her-2/neu growth factor receptor has intrinsic tyrosine-kinase activities that autophosphorylates and phosphorylates its dimerization partners, resulting in simultaneous activation of several signal transduction pathways and leading to the activation of the PI3K-Akt cell survival pathway and mitogen-activated protein kinase pathways that induce mitogenesis, cell proliferation, cell survival, and genomic instability. Amplification or overexpression of Her-2/neu or both have been detected in about 30% of human breast cancers and in many other types of human malignancies. In addition, overexpression of HER-2/neu in breast cancer has been associated with poor overall survival and has been shown preclinically to enhance malignancy and the metastatic phenotype.[67-69] Although discrepancies exist among different reports, Her-2/neu overexpression seems to have induced chemoresistance in several preclinical studies and a few clinical observations.[69] The predictive value of Her-2/neu overexpression and chemoresistance has been demonstrated in breast and ovarian cancer patients. In general, tumor cells overexpressing Her-2/neu are intrinsically resistant to DNA-damaging agents such as cisplatin. Although the molecular mechanisms by which Her-2/neu induces drug resistance are not yet established, there is evidence that this may be a consequence of altered cell cycle checkpoint and DNA repair mechanisms and dysregulation of apoptotic pathway(s)[67,69] (♣ please refer to III.6 and chapters by Yu et al in this book). Drug-induced apoptosis depends on the balance between cell cycle checkpoints and DNA repair mechanisms.[51] Blockade of Her-2/neu signaling using Her-2/neu antagonists, dominant negative mutants, or chemical inhibitors of Her-2/neu tyrosine kinase activity induces cell cycle arrest, inhibits DNA repair, and (or) promotes

apoptosis.[22,68] Less understood are downstream signal transduction cascades by which Her-2/neu affects these regulatory mechanisms.

P53

The p53 tumor suppressor gene is the most frequently mutated gene in human tumors, and loss of p53 function can both disable apoptosis and accelerate tumor development in transgenic mice. In addition, functional mutations or altered expression of p53 upstream regulators (such as ATM, Chk2, MDM2, and $p14^{ARF}$) and downstream effectors (PTEN, Bax, Bak, and Apaf-1) are also common in human tumors, including human breast cancer. Mutations in p53 or in the p53 pathway can produce the multidrug resistance phenotype in vitro and in vivo, and reintroduction of wild-type p53 into p53-null tumor cells can reestablish chemosensitivity.[48,70-73] Direct DNA damage that caused by most DNA-damaging drugs and UV or ionizing radiation and overexpression of oncoproteins (e.g., E1A, ras, myc) can activate the intrinsic apoptotic pathway.[52] As a sensor of cellular stress, p53 is a critical initiator of this pathway. P53 can transcriptionally activate pro-apoptotic Bcl-2 family members (such as Bax, Bak, PUMA, and Noxa) and repress anti-apoptotic Bcl-2 proteins (e.g., Bcl-2, Bcl-XL) and IAPs (e.g., Survivin). Additionally, p53 can also transactivate other genes that may contribute to apoptosis, including tumor-suppressor PTEN, apoptosis regulators or inducers such as Apaf-1, PERP, p53AIP1, CD95 and TRAIL receptors (TRAIL-R2/DR5), and genes that leading to an increase in reactive oxygen species (ROS).[71,72,74]

Bcl-2 Family Proteins

As discussed above, Bcl-2 family proteins play a pivotal role in the regulation of the intrinsic apoptotic pathway as guardians of the mitochondria, since these proteins localize particularly to the mitochondrial membrane.[75] Mutations or altered expression of pro-apoptotic or anti-apoptotic Bcl-2 family proteins can drastically alter drug response in experimental systems.[76,77] In addition, several clinical reports have provided support that a high expression level of anti-apoptotic Bcl-2 proteins confers a clinically important chemoresistant phenotype on cancer cells. Likewise, reduced expression of pro-apoptotic Bax levels has been associated with poor response to chemotherapy and shorter overall survival for patients with breast cancers, whereas enhanced expression of Bax protein correlated with a good response to chemotherapy in vivo.[76,78] In addition, chemotherapeutic drugs exert their effect in part by modulating the expression of several members of the Bcl-2 family proteins.[79] For example, paclitaxel, doxorubicin, and thiotepa upregulate several pro-apoptotic Bcl-2 proteins and downregulate anti-apoptotic Bcl-2 proteins. Blockade of Bcl-2 activity or expression by either an anti-Bcl-2 single chain antibody (anti-Bcl-2 sFv) or a novel Bcl-2/Bcl-XL bi-specific anti-sense oligonucleotide has demonstrated remarkable effect in enhancing drug-induced cytotoxicity in breast cancer cells.[79,80]

P-glycoprotein and MDR

Expression of the multidrug resistance gene (mdr) is one of the most-studied potential mechanisms underlying multidrug resistance. The human mdr gene family encompasses two homologous members, the first of which, called the mdr1 gene, is the best characterized so far. The human mdr1 gene encode a membrane P-170 glycoprotein that, on the basis of its structure, is considered to act as a drug-efflux pump excreting various drugs from cells. Resistance results because increased drug efflux lowers intracellular drug concentrations.[81,82] Analysis of 31 reports published between 1989 and 1996 found that 41% of breast tumors expressed P-170 glycoprotein. P-170 expression increased after therapy and was associated with a greater likelihood of treatment failure, although there was considerable inter-study variability.[2,82] In addition to actively effluxing chemotherapeutic drugs, the P-170 glycoprotein can also protect cells against apoptosis mediated by the death receptor pathway, UV irradiation, and serum starvation.[2,52]

Ceramide

Ceramide, or N-acyl-sphingosine, has been implicated in the acquired drug resistance in breast cancer. Ceramide is a metabolite of sphingomyelin hydrolysis by neutral or acidic sphingomyelinases. Sphingomyelin is the most abundant lipid in the plasma membrane of mammalian cells. Ceramide functions as a second messenger to signaling cascades that promote cell differentiation, proliferation, senescence, and apoptosis.[83] Ceramide is produced in response to a wide range of stimuli, including a long list of chemotherapeutic agents. Several lines of evidence have linked the failure of ceramide production to chemotherapy resistance, including the lack of a ceramide response in drug-resistant cell lines and the abrogation of paclitaxel-induced apoptosis by blocking ceramide synthesis.[79,83] In addition, protein phosphatases activated by ceramide, such as protein phosphatase 2A (PP2A), have been shown to promote inactivation of a number of anti-apoptotic molecules or pro-growth regulators, including Bcl-2, PKC, and Akt.[51,83]

PI3K/Akt Pathway

The PI3K-Akt signaling pathway, a major signaling component downstream of growth factor receptor tyrosine kinases, regulates fundamental cellular processes such as cell survival, growth, and motility—that are critical for tumorigenesis.[84] Indeed, aberrant activation of the PI3K-Akt pathway has been widely implicated in many cancers. For example, the serine/threonine kinase Akt and its family members Akt 2 and 3 are amplified or their activity is constitutively elevated in human carcinomas such as breast, pancreatic, ovarian, brain, prostate, lung, and gastric cancers.[85,86] As a direct downstream target of PI3K, Akt is a key oncogenic survival factor that can phosphorylate and inactivate a panel of critical pro-apoptotic molecules, including Bad, caspase-9, the Forkhead transcription factor FKHRL1 (known to induce expression of pro-apoptotic factors such as Fas ligand), GSK3-β, ASK1 (apoptosis signal-regulating kinase-1), cell cycle inhibitors p21 and p27, and tumor-suppressor TSC2.[71,84,87-93] In addition, we and others found that Akt can also inactivate p53 through phosphorylation and nuclear localization of MDM2.[88,89] Blockage of the Akt pathway by a genetic approach using a dominant-negative Akt (DN-Akt) construct resulted in p53 stabilization and accumulation and had a synergistic effect with the genotoxic anti-cancer drug etoposide in inducing apoptosis in NIH3T3 cells both in vitro and in vivo.[88] Numerous groups have reported the effects of chemotherapy on Akt activity in tumor cell lines. These studies showed that the administration of chemotherapeutic agents commonly results in reduced activity of the PI3K/Akt pathway. Although in certain cases chemotherapy agents increased Akt activity, in most cases such increases in Akt activity were transient and were followed by subsequent decreases in activity.[94,95] In addition, studies performed in vitro and in vivo combining small molecule inhibitors of the PI3K/Akt pathway with standard chemotherapy have successfully attenuated chemotherapeutic resistance.[85,96-98]

NF-κB

NF-κB is a multi-subunit nuclear transcription factor that regulates several cellular functions, including cell growth, differentiation, development, adaptive response to redox balance, and apoptosis.[99] In its inactive form, NF-κB resides in the cytoplasm bound to inhibitory IκB proteins that shield the DNA binding site. External stimuli, such as pro-inflammatory agents, infectious agents, stress, and chemotherapeutic drugs, activate NF-κB by phosphorylation and subsequent degradation of IκB proteins by the IκB kinase (IKK), whereupon NF-κB is transported into the nucleus and transcription of the target genes occur. As a nuclear transcription factor, NF-κB target genes including several anti-apoptotic proteins, e.g., the IAP family of caspase inhibitory proteins, TRAF1 and TRAF2 (which are thought to repress caspase-8 activation), Bfl1/A1, Bcl-XL, FLIP, and inducible nitric oxide synthetase. Interestingly, NF-κB also controls promoter activation of certain pro-apoptotic factors, such as CD95 (Fas) and CD95 ligand (FasL) and TRAIL receptors (TRAIL-R1 and -R2).[99,100] Whether NF-κB targets

pro- or anti-apoptotic genes depends on the stimulus-specific signaling pathway activated. Administration of certain types of anticancer drugs induced NF-κB transcriptional activation, whereas inhibition of NF-κB in parallel with chemotherapy strongly enhanced the cytotoxic effect of chemotherapy.[79,80,100] Thus, NF-κB may play an important role in inducible chemoresistance, and inhibition of NF-κB may confer sensitivity to cytotoxic anticancer drugs.

Structures, Biochemical Features, and Associated Cellular Proteins of E1A

The adenovirus type 5 (Ad5) E1A encodes two differentially spliced 12S and 13S mRNAs that give rise to 243 and 289 residue (243R, 289R) proteins, respectively. Each is comprised of two exons and, as splice joining exons 1 and 2 of the 12S mRNA is in frame with the spliced 13S transcript, the encoded proteins are identical apart from a central 46 residue region present in the larger product.[101,102] There are three minor E1A mRNA transcripts from Ad5 (11S, 10S, and 9S) that are expressed primarily at later stages of infection, and their functions are largely unknown.[101,103] The roles of the major 12S and 13S E1A products have been studied extensively and their importance in sensitization to apoptosis induced by anticancer drugs is well-documented and will be discussed here.

Both major E1A products are nuclear, highly acidic, and extensively phosphorylated transcription factors that exert their effects through their functional domains, which are highly conserved in virtually all human adenoviruses and in many adenoviruses of other animal species. These regions in Ad5 include conserved regions 1 and 2 (CR1 and CR2) located in Ad5 between residues 40 to 80 and between residues 120 to 139, respectively, and CR3 is located between residues 140 to 185. All three are encoded within exon 1 of the 289R product. CR1 and CR2 are present in both 289R and 243R; however, CR3 is unique to 289R, as it represents the 46 residues eliminated by splicing of the 12S mRNA[101] (Fig. 1). In addition, a region at the amino terminus, which is not highly conserved, is also of great importance. The first E1A-binding proteins to be detected were pRb tumor suppressor and the related proteins p107 and p130, and a 300-kDa transcriptional coactivator/signal integrating protein p300 and CBP, which is a relative of the cyclic AMP responsive element binding (CREB) protein.[101,104] In addition to pRb and p300/CBP, E1A also interacts with a 400-kDa protein doublet through its amino terminus 25-36 residues, which was recently characterized as a strong SWI2/SNF2 homology, p400, and TRRAP/PAF400, a myc-associated, transcriptional coactivator (TRRAP, transactivation/transformation-domain-associated protein) as well as a component of the p/CAF histone acetyltransferase complex (PAF400).[102] The E1A-p400 complex interaction is mainly linked to the E1A transformation mechanism and is not the focus of the current review.

Binding of the pRb family of proteins occurs primarily through a conserved binding site Leu-X-Cys-X-Glu found in the CR2 domain; however, a minor but critical contact is also made with a portion of CR1. Binding of p300/CREB/p400 requires the amino terminus and a region of CR1. P300 and CBP possess endogenous histone acetyl transferase (HAT) activity, which can be directly inhibited by E1A.[104,105] E1A may serve as a substrate for p300 and CBP to repress the HAT activity of p300/CBP, as it can be acetylated at Lys-239 by p300 and P/CAF.[105,106] The CR3 domain interacts with a number of transcription factors, including the TATA-binding protein (TBP), a critical component of the basal transcription complex, and upstream factors such as ATF members, Sp1, and c-Jun. A relatively uncharacterized 48-kDa proteins, termed CtBP, bind to a region encoded by exon 2, just adjacent to the carboxy terminal nuclear localization signal. CtBP is a transcriptional corepressor that was identified originally by its interaction with a conserved PXDLS motif near the carboxyl terminus of E1A.[107] Various repressor proteins that also contain the PXDLS motif recruit CtBP to promoters, where it represses transcription by subsequent recruitment of histone deacetylases (HDACs).[107] It has been shown that mutants of E1A that mimic the effect of acetylation of the Lys239 at the PXDLS motif of E1A by p300/CBP and P/CAF make E1A defective in CtBP binding. These E1A mutants that mimic the effect of acetylation were also defective in repressing

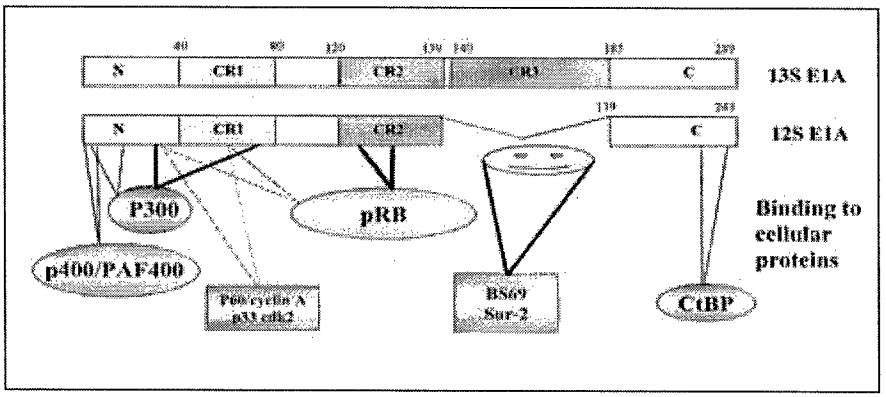


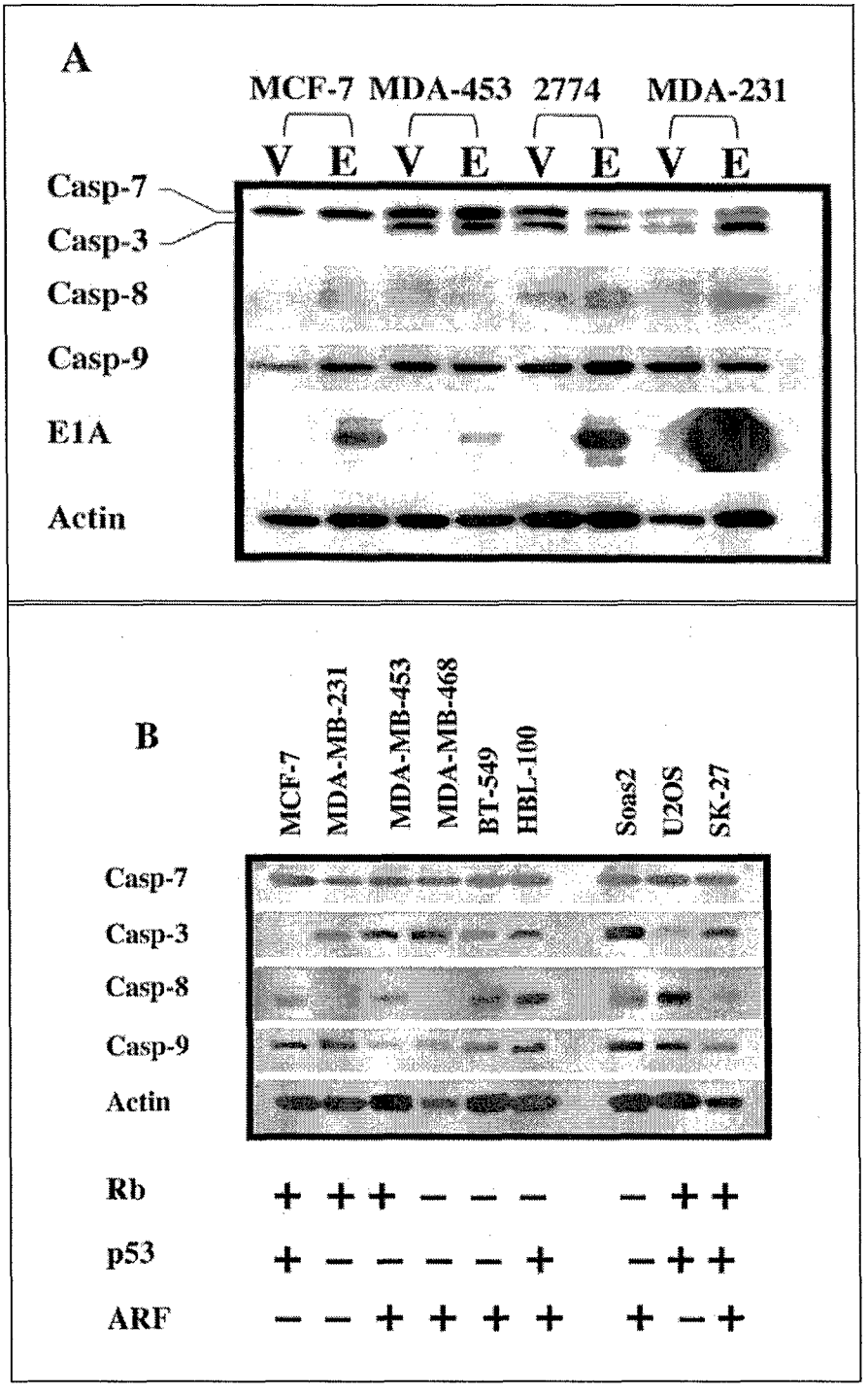
Figure 1. Map of E1A functional domains in 13S and 12S E1A proteins: conserved regions (CR) 1, 2, 3 and consensus sites for binding to cellular proteins.

CREB-stimulated (CBP-dependent) transcriptional activation under certain conditions, suggesting that E1A-mediated transcriptional repression may require interaction with CtBP. In addition, the tumor-suppressor proteins pRb and BRCA1 also recruit CtBP through an adaptor protein CtIP, indicating that CtBP might be involved in the transcriptional repression and/or apoptosis that is regulated by these proteins.[107]

E1A products interact with pRb, p107, and p130 via the major binding site in the CR2 domain and the N-terminal portion of the CR1 domain plays an auxiliary role; however, it is the CR1 domain that competes directly with E2F for access to the pRB pocket, i.e., the E1A CR1 domain can inhibit E2F-pRb complex formation and E1A, by binding with pRB through CR1, releases E2F.[101,108] E2F was originally described as a factor regulating adenovirus E2 transcription; however, it soon became apparent that it is of general importance in the expression of genes encoding DNA synthetic enzymes and regulators of the cell cycle and apoptosis.[109,110] E2F is a sequence-specific, DNA-binding transcription factor that exists as a family of heterodimers containing one of six E2F proteins bound to either DP1 or DP2.[110] A major function of the pRb tumor-suppressor family is to bind to and inactivate E2F. E2F-1, -2, and –3 heterodimers bind to pRb; E2F-4 to pRb, p107 and p130; and E2F-5 to p130 only. E2F6 does not interact with the pRb family of pocket proteins and functions as a negative regulator of E2F-dependent transcription via complexing with chromatin modifiers.[109-111] Binding of E2F to pRb family members involves the "large binding pocket" that overlaps the region required for E1A protein binding. When E1A proteins bind to pRb and its family members, the viral protein competes with E2F for occupancy of the pocket domain, dissociating E2F from the tumor suppressor proteins. When E2F is released, it can activate transcription from promoters that contains its binding site.[108] Exogenous expression of E2F1, E2F2 or E2F3 in quiescent cells results in S-phase entry. In addition, ectopic expression of E2F1 leads to apoptosis in tissue culture cells and transgenic mice.[109]

Chemosensitization by E1a

Ever since the landmark discovery by Lowe and coworkers in 1993[28,40] that the adenovirus E1A can sensitize fibroblasts to apoptosis induced by ionizing radiation and anticancer drugs in a p53-dependent manner, a variety of mechanisms for E1A-mediated chemosensitization have been proposed. It is now recognized that both p53-dependent and –independent pathways, $p14^{ARF}$-dependent and –independent pathways, and additional pathways may all contribute to E1A-mediated chemosensitization in different cellular contexts. Summarizing these


A
MCF-7 MDA-453 2774 MDA-231
V E V E V E V E
Casp-7
Casp-3
Casp-8
Casp-9
E1A
Actin
B
MCF-7
MDA-MB-231
MDA-MB-453
MDA-MB-468
BT-549
HBL-100
Soas2
U2OS
SK-27
Casp-7
Casp-3
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p53 + − − − − + − + +
ARF − − + + + + + − +

Figure 2. Expression of caspase–3, –7, –8, and –9 proenzymes in human cancer cells. (*A*), Caspase proenzyme expression in E1A stable cells established in human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-453; and ovarian cancer cell line 2774. V: vector control; E: E1A stable cells. (*B*), Procaspase enzyme expression in Rb wild-type breast cancer cells MCF-7, MDA-MB-231, MDA-MB-453; normal foreskin fibroblast cell SK-27; and osteosarcoma U2OS cells versus Rb-deficient cells (MDA-MB-468, BT-549, HBL-100, and Soas2). SK-27, U2OS, and Soas2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Mouse monoclonal antibodies against human caspase–3 and –7 were from Transduction Laboratories (1:1000; C31720, Lexington, KY) and BD PharMingen (1:1000; 66871A, San Diego, CA), respectively. Rabbit polyclonal antibodies against human caspase–8 and –9 were from Santa Cruz Biotechnology (1:500; SC-7890/H-134, Santa Cruz, CA) and Cell Signaling Technology (1:500; #9502, Beverly, MA), respectively. Figure .(A) was adapted with permission from Ref. 37 (Liao Y & Hung MC: *Mol. Cell Bio.*, 2003;23:6836).

different molecular mechanisms underlying E1A-mediated chemosensitization is the major focus of this review.

Trancriptional Activation of Pro-apoptotic Molecules: The E2F-1 Pathway

Earlier studies in mouse embryo fibroblasts (MEF) with wild-type p53 or p53 deletion demonstrated that the E1A-mediated sensitization to apoptosis induced by ionizing radiation and several chemotherapeutic agents (5-fluorouracil, etoposide, and doxorubicin) depends on the expression of functional p53 tumor suppressor, as treatment with E1A-expressing cells lacking p53 had little or no effect on viability.[28,40,41,42,112-118] The stability of p53 protein is increased in cells expressing E1A. Further study showed that stabilization and accumulation of p53 by E1A is through the $p19^{ARF}$ tumor suppressor, since the ability of E1A to induce p53 and its transcriptional targets is severely compromised in ARF-null cells.[44,117,119] ARF ($p19^{ARF}$ in the mouse, $p14^{ARF}$ in human cells) is encoded by an Alternative Reading Frame of the Ink4A tumor suppressor locus. It binds to MDM2, an E3 ubiquitin ligase of p53, and inhibts its E3 ligase activity, thereby allowing p53 to escape MDM2-mediated ubiquitination and degradation and a consequent accumulation of active p53.[120] Reports from White's group showed that E1A inhibited MDM2 transactivation without affecting the expression of p21 or Bax, which resulted in a high level of p53 accumulation and apoptosis by a p300-dependent mechanism.[121] Since p300 is required for MDM2 induction by p53 and subsequent inhibition of p53 stabilization, inhibition of p300 by E1A results in p53 stabilization and causes apoptosis.[121]

A recent report from our group demonstrated that an alternative mechanism may also contribute to the stabilization of p53 by E1A.[122] By screening a yeast two-hybrid library, an MDM2-related p53 binding protein, MDM4, was identified as a direct binding partner of E1A. The NH2-terminal region of MDM4 and the CR1 domain of E1A were required for the interaction between MDM4 and E1A.[122] Since the CR1 domain of E1A is involved in binding with p300, it is not clear whether p300 is also involved in the interaction between MDM4 and E1A. However, we were able to show a tri-complex formation among E1A, MDM4, and p53 that resulted in the stabilization of p53 independent of the $p14^{ARF}$ protein expression.[122] E1A did not affect the p53-MDM2 interaction, but it inhibited MDM2 binding to MDM4, resulting in decreased nuclear exportation of p53.[122] As discussed above, p53 can initiate apoptosis by transcriptionally activating pro-apoptotic Bcl-2 family members and repressing anti-apoptotic bcl-2 proteins and IAPs. In addition, p53 can also transactivate other genes that contribute to apoptosis, including PTEN, Apaf-1, PERP, p53AIP1. Among these p53 target genes, elevated expressions of Apaf-1 and Bax have been reported in response to E1A and partly contributed to E1A-mediated chemosensitzation.[123,124] In baby rat kidney cells, the activity of p53 as a transcription factor is directly correlated with the ability of E1A to induce apoptosis. Whether other p53 target genes are also altered in E1A-expressing cells needs further investigation.

Disrupting RB-E2F heterodimers and subsequent releasing of free E2F-1 by E1A has been suggested to contribute to ARF induction, which is also consistent with the possibility that ARF is an E2F-response gene. Enforced expression of E2F induces $p19^{ARF}$ and, conversely, ARF null cells are resistant to E2F-1-induced apoptosis.[109,110,118,125-130] In addition to the ARF protein, E2F1 can directly activate transcription of p73, a member of the p53 family, in turn leading to activation of p53-responsive target genes and apoptosis. Disruption of the p73 function, by dominant negative p73 mutants or by gene targeting, inhibits E2F-1-induced apoptosis.[109,131] E2F-1 also directly activates the expression of the Apaf-1 gene.[132] Induction of E2F-1 activity results in an increase of mRNA and protein levels of Apaf-1 and a concomitant activation of caspase-9, -3, and -6 and possibly caspase-7. E2F-1-induced apoptosis is significantly reduced by gene disruption of Apaf-1. In addition, RB null embryos exhibit increased levels of Apaf-1, suggesting that Apaf-1 is required for apoptosis induced by E1A-mediated pRb deficiency and subsequent E2F-1 activation.[109,132] Apaf-1 is a also direct transcriptional target of p53, raising the possibility that its transactivation by E2F-1 is indirect. However, E2F-1 binds the Apaf-1 promoter and transactivates the Apaf-1 gene in cells lacking p53, and E2F-1 also activates the deleted version of the Apaf-1 promoter that is not activated by p53, suggesting Apaf-1 is a direct target of E2F-1.[109] In addition to ARF, p53, p73, and Apaf-1, a recent study from Lowe's laboratory also demonstrated that pro-caspase-3, -7, -8, and –9 can also be direct transcriptional targets of E2F-1 and disrupting pRb-E2F-1 complex by E1A, loss of pRb expression by genetic approach, or enforced E2F-1 expression results in the accumulation of caspase proenzymes and sensitization to apoptosis after the release of cytochrome *c* in fibroblasts.[130] Although a number of recent studies have demonstrated that RB inactivation or E2F-1 overexpression leads to apoptosis that is inhibited by loss of p53 but not by loss of ARF, E1A induces expression of these pro-caspase enzymes in cells deficient in either p53 or ARF, suggesting that E1A regulates pro-caspase expression through a p53-independent mechanism.[130]

Interestingly, studies using E2F-1 mutants have demonstrated that although its DNA-binding activity is required, transcriptional transactivation is not necessary for the induction of apoptosis by E2F-1.[129,133,134] Regardless of the fact that expression of E1A induced accumulation of caspase pro-enzymes in human normal diploid fibroblasts, screening the expression levels of caspase pro-enzymes in E1A–expressing human carcinoma cells with epithelial origin, including breast cancer MDA-MB-231, MDA-MB-453, MCF-7, and ovarian cancer 2774, did not observe a unanimous increase of these caspase pro-enzymes.[37] This again suggests that transcriptional upregulation of these caspase pro-enzymes in human cancer cells may not be as critical as that in the normal fibroblast cells for E1A–mediated sensitization to apoptosis. The discrepancy between normal diploid fibroblasts and epithelial carcinoma cells in E1A–mediated sensitization to apoptosis may reflect the nature of the intrinsic difference between normal fibroblasts and carcinoma cells. In addition, the pRb pathway is functionally inactivated in most human cancers; however, loss of pRb expression in human cancer cells may not necessarily result in an increased expression of caspase pro-enzymes, according to our screening of human breast cancer cells and normal skin cells and fibrosarcoma cell lines with different genetic backgrounds in expression of p53, ARF, and pRb proteins (Fig. 2). Intriguingly, noticeable induction of apoptosis in anticancer drug-treated cells expressing E1A mutant incapable of binding to pRB was also observed recently by Chattopadhyay et al,[135] and they also failed to detect the release of E2F-1 in their system, suggesting that additional mechanisms may be involved in E1A-mediated sensitization to apoptosis in human cancer cells.

Transcriptional Inactivation of Receptor Tyrosine Kinases: The Her-2/neu and Axl Pathway

Earlier studies from our group demonstrated that E1A may function as a tumor suppressor for Her-2/neu-overexpressing cancer cells by repressing the expression of Her-2/neu at the transcriptional level.[7-9,17,22,32,136-139] The Her-2/neu promoter contains several positive ele-

ments that require the p300/CBP coactivator proteins, which are inhibited by direct E1A interaction. The NH2-terminus and CR1 domain of E1A that is required for E1A to bind with p300 is also required for transcriptional repression of Her-2/neu.[140,141] When introduced into rat B104-1-1 cells transformed by the mutated rat Her-2/neu gene, E1A gene products suppressed the transformed phenotypes and inhibited the metastatic potential of the B104-1-1 cells. Similar results were also observed in SKOV3.ip1 human ovarian cancer cells that overexpress Her-2/neu. The E1A-expressing ovarian cancer cell lines showed decreased p185Her-2/neu expression and reduced malignancy, including a decreased ability to induce tumors in *nu/nu* mice. In addition, preclinical studies using either liposome- or adenovirus-mediated E1A gene transfer found inhibited tumor growth of Her-2/neu-overexpressing breast cancer cells injected into the mammary fat pads of mice and prolonged animal survival compared with controls.[32,137-139] As discussed above, breast tumors that overexpress Her-2/neu are less responsive to treatment with various anticancer agents, such as cyclophosphamide, methotrexate, 5-fluorouracil, epirubicin, paclitaxel and docetaxel, and patients with cancers that overexpress Her2/neu are associated with unfavorable prognosis, shorter relapse time, and lower survival rate.[69,142-144] Therefore, we were prompted to test whether downregulation of Her-2/neu expression by E1A conferred an enhanced sensitivity to anticancer drugs in Her-2/neu overexpressing breast cancer cells. When the E1A gene was transferred into two human breast cancer cell lines that overexpress Her-2/neu (MDA-MB-453 and MDA-MB-361), levels of Her-2/neu expression in the E1A transfected cells were reduced. The cell proliferation assay and soft agar colony-formation assay indicated a synergistic growth-inhibitory effect following treatment with the combination of E1A gene and paclitaxel in breast cancer cells that overexpress Her-2/neu. A similar synergistic effect was also observed recently following liposome-mediated systemic delivery of the E1A gene via the mouse tail vein in Her-2/neu over-expressing human breast cancer xenograft model.[145] The data indicated that E1A can sensitize Her-2/neu-overexpressing breast cancer cells to paclitaxel through E1A-mediated Her-2/neu repression. This finding has important clinical implications for the development of a novel therapeutic strategy by combining paclitaxel chemotherapy with E1A gene therapy for the treatment of Her-2/neu-overexpressing breast cancers, and a Phase I/II clinical trial is currently underway to determine the therapeutic efficacy of this combination strategy in such cancers. An earlier report by Sabbatini et al demonstrated that expression of Her-2/neu and c-Ha-ras oncogenes induces expression of the P-glycoprotein MDR1 mRNA and confers resistance to doxorubicin in immortalized normal epithelial MCF-10A breast cells, which are negative for the expression of P-glycoprotein.[146] In studies of low Her-2/neu-expressing MDA-MB-435 breast cancer cells, Yu et al observed that expression of Her-2/neu in the cells did not induce P-glycoprotein expression and blocking MDR1 by thioradazine did not sensitize these transfectants to paclitaxel treatment, suggesting that overexpression of Her-2/neu leads to intrinsic paclitaxel resistance independent of the MDR1 mechanism.[142] Further studies by Yu et al suggested that Her-2/neu acts synergistically with MDR1 to confer a higher degree of paclitaxel resistance.[147] It is yet to be determined whether expression of E1A also affects MDR1 expression, in addition to down-regulation of Her-2/neu, for E1A-mediated chemosensitization.

Using a tyrosine kinase differential display (TK-DD) approach, we found that Axl, another oncogenic receptor tyrosine kinase, can also be transcriptionally repressed by the expression of E1A. Similar to the observed downregulation of Her-2/neu, transcriptional repression of Axl by E1A also sensitized cells to apoptosis induced by serum deprivation.[148] Further elucidation of the molecular mechanisms underlying E1A-mediated inhibition of Axl led to the finding that Akt, a critical Axl downstream molecule, was inactivated as a result of E1A expression (see detailed discussions below).[149] However, when we enforced Axl expression in the E1A-expressing cells, E1A-mediated sensitization to apoptosis was abrogated and Akt was reactivated in the presence of the Axl ligand Gas6.[149] These studies suggest that E1A can sensitize cells to apoptosis using alternative pathways in addition to its function in repressing Her-2/neu expression. Indeed, we and others have reported that sensitization to the cytotoxic effects of anticancer drugs

(such as cisplatin, adriamycin, gemcitabine, methotrexate, and paclitaxel) by the expression of E1A was also achieved in cells that do not overexpress Her-2/neu.[30,37,39,150-152] In one study, expression of 12S E1A alone or 12S plus 13S E1A did not affect Her-2/neu expression in cancer cell lines expressing a low level of Her-2/neu; however, expression of E1A not only severely reduced the anchorage-independent and tumorigenic growth of these cell lines but also sensitized these cells to the cytotoxic effects of the anticancer drugs.[30] In an E1A-expressing murine melanoma cell system that does not overexpress Her-2/neu, expression of E1A repressed tumor growth in vivo and sensitized the cells to apoptosis induced by either γ-radiation or cisplatin.[150] In addition, recent reports from our group showed that E1A mediated chemosensitization in the low Her-2/neu-expressing breast cancer cell lines MCF-7 and MDA-MB-231 in vitro and in an orthotopic animal model in vivo in a systemic gene therapy setting.[37,39,152] We had previously shown that E1A, through downregulation of Her-2/neu, sensitized cellular response to paclitaxel-induced apoptosis in Her-2/neu overexpressing cells.[32,137,138] In those studies, we could not detect E1A-mediated chemosensitization in the low Her-2/neu-expressing cells. The major reason for this discrepancy was due to the paclitaxel concentrations tested. The Her-2/neu-overexpressing cancer cells are resistant to paclitaxel at a dosage of 10.0 μM; in the presence of E1A, however, they became sensitive at a paclitaxel dose of 1.0 μM. Whereas, the low Her-2/neu-expressing cells, such as MDA-MB-231and MDA-MB-435, are much more sensitive to paclitaxel, even at a concentration of 0.1 μM. Therefore, in the previous studies, the dose of paclitaxel applied was too high (1 μM) to detect the in vitro E1A-mediated paclitaxel sensitization that was found in the later study (0.01 μM). Therefore, transcriptional repression of Her-2/neu expression is only one of the many mechanisms underlying E1A-mediated chemosensitization and tumor suppression.

Targeting the Intracellular Signaling: The Akt/ASK-MEKK3/p38 Pathway

The breast cancer cell lines MCF-7 and MDA-MB-231 express low levels of Her-2/neu, however, expression of E1A enhanced their sensitivity to apoptosis induced by various anticancer drugs. Because MDA-MB-231 cells express no functional p53 and ARF, whereas MCF-7 cells express an undetectable level of Axl mRNA and protein,[153] we therefore prompted to explore whether additional mechanisms may contribute to E1A-mediated chemosensitization in these cell systems. To determine whether apoptosis-related kinases are involved in E1A-mediated sensitization to apoptosis, we examined the phosphorylation status of the well-known kinases representing different signaling pathways involved in the regulation of apoptosis[154] – p38, Akt, Erk, and JNK – in E1A-expressing MDA-MB-231 and MCF-7 cells (231-E1A and MCF-7-E1A) versus vector-transfected cells (231-Vect, MCF-7-Vect). The phosphorylation levels of JNK1/2 kinases were unchanged in E1A-expressing versus vector control cells in the absence of drug stimuli, whereas upon treatment with paclitaxel, the JNK1/2 kinases were transiently activated and then reduced to the basal level. Kinetically, phosphorylation of JNK1/2 does not correlate with paclitaxel-induced Bcl-2 phosphorylation and PARP cleavage, two hallmarks of paclitaxel-induced apoptosis; therefore, it is unlikely that JNK is the critical player in E1A-mediated chemosensitization in these cell systems. Although the basal phosphorylation level of Erk1/2 was increased in E1A-expressing cells compared with their respective vector controls, blocking of Erk1/2 activity by a specific MEK (a direct upstream kinase of Erk1/2) inhibitor PD98058 alone drastically enhanced spontaneous apoptosis in the absence of paclitaxel in E1A-expressing cells but not in the vector controls, suggesting that Erk is a critical survival factor in stable E1A-expression cells and that elevated Erk1/2 activity do not contribute to E1A-mediated chemosensitization in our system (Liao et al, unpublished data). Elevated expression of phosphorylated p38 was observed in both E1A-expressing MCF-7 and MDA-MB-231 cells, whereas the total level of p38 protein was similar in cells with or without expression of E1A. To test whether alteration of the kinase activity of p38 played a role in E1A-mediated sensititization to drug-induced apoptosis, we compared the kinetics of phosphorylation of p38 with paclitaxel–induced apoptosis in 231-E1A cells, using PARP cleavage

and Bcl-2 phosphorylation as apoptotic cell death markers. Kinetically, PARP cleavage and Bcl-2 phosphorylation occurred after increased p38 phosphorylation in 231-E1A cells upon exposure to paclitaxel, suggesting that elevated p38 phosphorylation may be associated with E1A-mediated sensitization to paclitaxel-induced apoptosis. To evaluate whether activation of p38 is required for E1A-mediated sensitization to paclitaxel, we tested whether blocking p38 activity will inhibit E1A-mediated sensitization in 231-E1A cells. We used a specific p38 inhibitor (SB203580) and an IPTG-inducible dominant-negative p38 (DN-p38) mutant to block p38 activation; as expected, blockade of p38 activation in 231-E1A cells by either the p38 inhibitor or the DN-p38 obviously compromised E1A-mediated sensitization to anticancer drug-induced apoptosis, suggesting that p38 activation is required for E1A-mediated sensitization to apoptosis.[37]

Unlike results for p38, the level of phosphorylated Akt was reduced in E1A-expressing 231-E1A and MCF-7-E1A cells. Kinetically, decreased levels of phosphorylated Akt correlated with paclitaxel-induced PARP cleavage and Bcl-2 phosphorylation in 231-E1A cells, suggesting that downregulation of Akt phosphorylation in E1A-expressing cells may also contribute to E1A-mediated chemosensitization. To determine whether downregulation of Akt activity is required for E1A-mediated chemosensitization to paclitaxel, we examined whether activation of Akt by transfection of a constitutively active Akt construct (CA-Akt) would inhibit paclitaxel-induced apoptosis in 231-E1A cells. Reactivation of Akt in E1A-expressing 231-E1A cells repressed p38 phosphorylation in the presence or absence of paclitaxel and reduced paclitaxel-induced apoptosis in the cells. In addition, we also observed that activation of p38 and inactivation of Akt represent a general cellular mechanism in response to different apoptotic stimuli in the presence or absence of E1A.[37]

In an attempt to elucidate whether downregulation of Akt and upregulation of p38 are separate events or are linked events, we sought to determine whether Akt might act upstream of p38. Blockade of Akt activity in MDA-MB-231 cells by wortmannin, a specific PI3K inhibitor, decreased Akt phosphorylation and increased p38 phosphorylation. Similar results were also obtained by using LY2049002, another PI3K inhibitor. These results indicated that Akt phosphorylation is required for repressing p38 activation, that is, that the former is upstream from the latter. This conclusion was further supported by our study on Akt1 knockout mouse embryo fibroblast (MEF) cells and myr-Akt1 transfected stable cells. In that study, we observed that the level of phosphorylated p38 was increased in Akt1 (–/–) MEF cells compared with that in Akt1 (+/+) and Akt1 (+/–) MEF cells. Furthermore, the phospho-p38 protein was undetectable in the myr-Akt1 stable cells, suggesting that activation of p38 is associated with inactivation of Akt or, in other words, that Akt represses p38 activation in a physiological condition (Fig. 3).

Recent reports have demonstrated that ASK1 and MEKK3 are substrates of Akt, and both kinases have been shown to be upstream kinases of p38,[91,155-157] suggesting that Akt may indirectly regulate p38 activity through repression of ASK1 or MEKK3 or both. To test this, we blocked the activity of either ASK1 or MEKK3 by using a kinase-dead, dominant-negative mutant of ASK1 (DN-ASK1) or MEKK3 (DN-MEKK3). As expected, blockade of either ASK1 or MEKK3 activity by DN-ASK1 or DN-MEKK3 repressed p38 phosphorylation and its kinase activity, as measured by phosphorylation of GST-ATF-2 fusion protein in a dose-dependent manner in Akt1 (–/–) MEF cells. This result suggests that Akt can inhibit p38 activation through repression of ASK1 and/or MEKK3 activation. Unlike what we expected, however, the combination of both DN-ASK1 and DN-MEKK3 did not results in an additional reduction of p38 kinase activity or phosphorylation of p38 in these MEF Akt1 (–/–) cells.[37] These data imply that alternative pathway(s) may exist in which Akt acts on p38, though low transfection efficiency may also contribute to the incomplete blockade of ASK1 and MEKK3 activity and subsequent p38 activation in the Akt1 (–/–) cells. The data established that E1A induced p38 activation by repressing Akt. A recent report by Yuan et al[158] demonstrated that, like Akt1, Akt2 can also inhibit p38 activation through phosphorylation of ASK1, which is

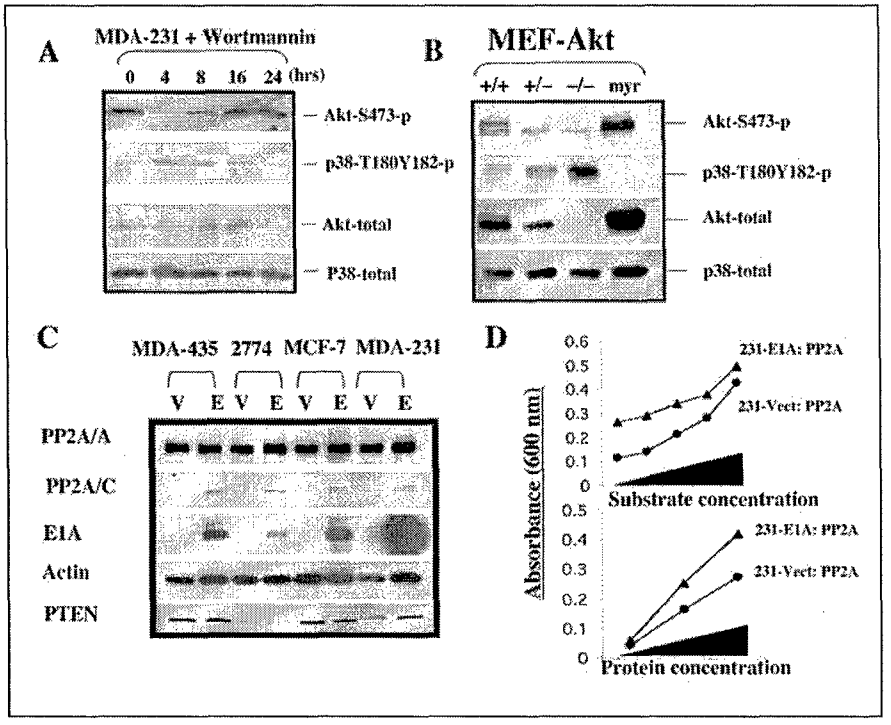

Figure 3. Inhibition of Akt activation by the PI3K inhibitor wortmannin or gene disruption of Akt1 results in p38 activation. (A) Stable E1A-expressing or parental MDA-MB-231 cells were serum starved for 24 hours before exposure to 0.1 μM wortmannin. (B) Expression of phospho-p38 and phospho-Akt in Akt1 knockout MEF cells and myr-Akt–transfected Rat1 cells. (C) E1A upregulates the expression of PP2A/C, the catalytic subunit of PP2A. (D) Expression of E1A upregulates protein phosphatase activity of PP2A. Figure .(A)–(B) and (C)–(D) were adapted with permission from Ref. 37 (Liao Y & Hung MC: *Mol. Cell Bio.*, 2003;23:6836) and Ref. 152 (Liao Y & Hung MC: *Cancer Res.* 2004;64:5938), respectively.

attributed to Akt2-mediated chemo-resistance; whether E1A also represses Akt2 phosphorylation needs further elucidation. We also observed that p38 activity is repressed in different types of human tumors, which is associated with enhanced Akt activation in human tumor tissue samples.[37]

Akt is a critical downstream target of PI3K and plays a pivotal role in positive regulation of cell survival and cell growth and also in negative regulation of apoptotic cell death, as summarized above.[78,84,86,87,159-163] Inhibition of Akt activation by E1A has important implications in terms of E1A-mediated sensitization to apoptosis. Akt regulates cellular survival through phosphorylation of downstream substrates that directly or indirectly control the apoptotic machinery. For example, phosphorylation of IKK by Akt releases NF-κB from inhibition by I-κB (an inhibitor of NF-κB) and increases transcription of pro-survival genes, such as inhibitors of caspases (c-IAP1 and c-IAP2), Bcl-2 homologues (A1/Bfl-1 and Bcl-xL), and adaptor molecules intercepting death signals (e.g., c-FLIP, TRAF-1, and TRAF-2). In return, TRAF-2 can augment TNF-α-induced NF-κB activation.[60,87,164-166] Earlier reports from our laboratory demonstrated that expression of E1A downregulated IKK and NF-κB activities, which was also attributed to E1A-mediated sensitization to apoptosis induced by γ-radiation, TNF-α and anticancer drug gemcitabine.[38,167,168] In addition, E1A has been shown to sensitize cells to

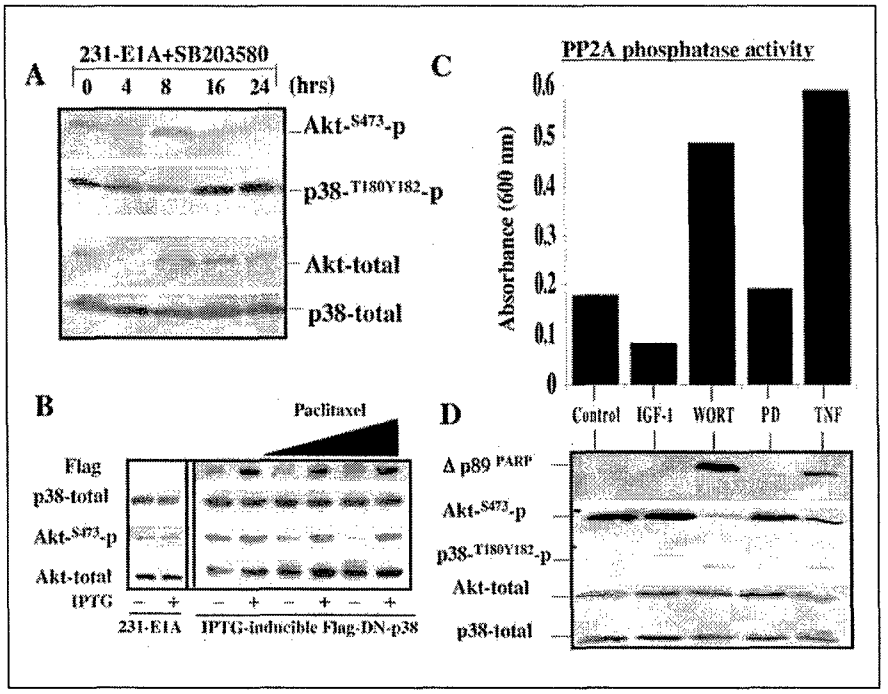

Figure 4. PP2A is involved in the cross-regulation between the Akt and p38 pathways. (A) Expression of phospho-Akt and -p38 in stable E1A-expressing or parental MDA-MB-231 cells that were serum-starved for 24 hours before exposure to 20.0 μM SB203580, a specific p38 inhibitor. (B). Repression of p38 activity by IPTG-inducible DN-p38 enhances Akt phosphorylation and abrogates E1A-mediated sensitization to paclitaxel in E1A-expressing cells in the presence of 5 μM IPTG for 24 hours. (C). Regulation of PP2A phoaphatase activity by IGF-1, wortmannin, and TNF-α in MDA-MB-231 cells. (D). PARP cleavage and Akt and p38 phosphorylation in MDA-MB-231 cells after exposure to IGF-1, wortmannin, and TNF-α. WORT: wortmannnin; PD: PD98058; Flag: anti-Flag tag. Figure (B) and (C)–(D) were adapted with permission from Ref. 37 (Liao Y & Hung MC: *Mol. Cell Bio.*, 2003;23:6836) and Ref. 152 (Liao Y & Hung MC: *Cancer Res.* 2004;64:5938), respectively.

TNF-α-induced apoptosis, by downregulating the expression of c-FLIP and preventing its induction by TNF-α.[169] Expression of c-FLIP has been reported to depend on the activity of PI3K/Akt pathway;[164,166] it is not yet clear whether E1A-mediated down-regulation of c-FLIP, IKK and NF-κB activities are resulted from E1A-mediated inhibition of Akt.

Expression of exogenous E2F-1 or induction of endogenous E2F-1 activity by inactivation of pRb was shown to downregulate TRAF-2 protein levels, thus leading to impaired TNF-receptor-mediated NF-κB activation in response to TNF-α, which does not require E2F-1-dependent transactivation.[134] A recent report further showed that E2F-1 can inhibit NF-κB activation by either stabilizing I-κB or competing with p50 for RelA/p65 binding,[170,171] which provide another mechanism for E1A-mediated inhibition of NF-κB activation, though it does not exclude the possibility that Akt may still be involved.

Studies from our group and several others have demonstrated that Akt can also regulate the stability of p53 by phosphorylation and nuclear localization of MDM2.[71,72,88,172] Phosphorylation of MDM2 by Akt promotes nuclear localization of MDM2 and its interaction with p300 and inhibits the interaction of MDM2 with $p19^{ARF}$, thus increasing p53 degradation through MDM2-mediated ubiquitination.[71,72,88,172] If E1A-mediated repression of Akt activ-

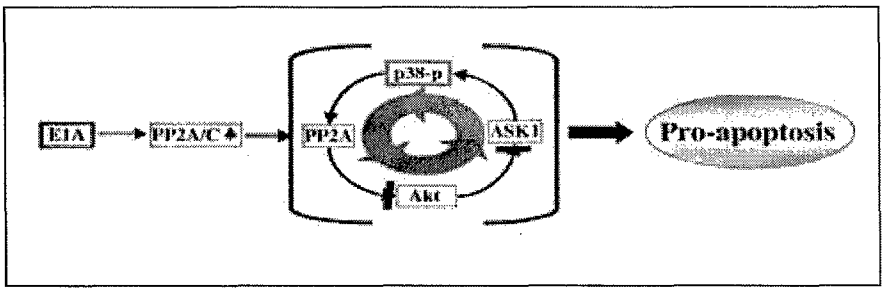

Figure 5. A feed-forward model for E1A-mediated sensitization to apoptosis.

ity could also affect p53 stability by this mechanism, it would be another E2F-1-independent pathway for p53 stabilization in E1A-expressing cells, and application of the dominant negative E2F-1 or the E2F-1-null MEF cell system may help to clarify this issue.

Duelli et al[173] have shown that E1A facilitates cytochrome *c* release from the mitochondria, thereby contributing to E1A-mediated sensitization to anticancer drugs; however, the mechanism by which E1A facilitates cytochrome *c* release is unclear. Akt is known to play an important role in maintaining mitochondria integrity and inhibiting the release of cytochrome *c*, and overexpression of Akt confers resistance to paclitaxel by inhibiting paclitaxel-induced cytochrome *c* release.[174] Akt also negatively regulates FasL transcription and translation by phosphorylating members of the Forkhead protein family. In addition, FasL transcriptional activation has been shown to depend on p38;[175] p38 is also involved in the regulation of cytochrome *c* release.[176] Therefore, E1A may alter mitochondrial potential by downregulating Akt and upregulating p38, thereby facilitating the release of cytochrome *c* or the expression of FasL upon treatment with chemotherapeutic drugs such as paclitaxel. Thus, in E1A-expressing cells, a relatively low concentration of drug would be sufficient to trigger apoptosis, regardless of the drug's primary target. Yet, it is not clear whether expression of E1A altered FasL expression.

Moreover, Schmidt et al demonstrated that expression of an active Akt suppresses chemotherapy-induced apoptosis by preventing mammary epithelial cells from undergoing anoikis (from the Greek word for "homelessness"),[177] another form of apoptotic cell death that occurs when epithelial cells lose contact with the extracellular matrix or bind through an inappropriate integrin.[16,18,178-182] Tumor suppressor PTEN has been shown to promote anoikis through its ability to repress Akt activation.[183-185] A recent report by Nagata et al[186] demonstrated that PTEN activation contributes to tumor inhibition by the Her-2/neu targeting antibody trastuzumab (Herceptin). Like constitutive activation of Akt, which confers resistance to chemotherapy and trastuzumab in breast cancer cells,[94] loss of PTEN also predicts trastuzumab resistance in patients due to deregulated PI3K-Akt activation and PI3K inhibitors can rescue loss of PTEN-induced resistance to trastuzumab. E1A is the first protein that has been reported to confer sensitivity to anoikis, and the ability to induce anoikis has also been linked to E1A-mediated tumor suppression and mesenchymal-epithelial transition (MET).[16,18,180,181] Therefore, it would be interesting to test whether down-regulation of Akt by E1A contributes in part to E1A-induced anoikis and MET.

Taken together, these findings indicate that many cellular proteins that interact with and/or are regulated by E1A, such as p53, caspase-8, caspase-9, Bax, NF-κB, p38, are also regulated by Akt. Although not tested, the critical apoptotic regulators Apaf-1 and Bcl-2 may be direct targets of Akt as they both contain the consensus Akt phosphorylation sites. In addition, phenotypes induced by expression of E1A, such as apoptosis, anticancer drug response, anoikis, MET, and tumor formation, are also subject to regulation by Akt. Thus, Akt is not only a pivotal cellular target for the development of anticancer drugs; it is also a critical target of E1A.

Targeting the Protein Phosphatase 2A (PP2A): A Feed-forward Model

Repression of Akt activation by E1A has also been demonstrated in normal fibroblast IMR90 and Cos-7 cells and linked to the sensitization to apoptosis induced by anticancer drug cisplatin. In addition, expression of E1A can inhibit insulin-mediated Akt activation in the Cos-7 cells.[36] We also observed a similar inhibition effect on Akt activation by insulin in the epithelial breast cancer cell lines MDA-MB-231 and MCF-7 cells, which stably express E1A (unpublished data). However, the mechanisms underlying E1A-mediated repression of Akt activation are not yet known. In Cos-7 cells, E1A was shown to directly repress basal and insulin-stimulated Akt phosphorylation without affecting the Akt protein expression.[36] This raises a question how can E1A, which is not a kinase, represses another kinase without affecting the protein expression or upstream factors or respective protein phosphatases? As discussed above, downregulation of Her-2/neu and/or Axl expression may contribute in part to repression of Akt activation by E1A in cells that overexpress Her-2/neu or Axl. Again, both MDA-MB-231 and MCF-7 cells express low levels of Her-2/neu, and MCF-7 cells have an undetectable level of Axl mRNA and protein.[153] We also failed to detect alterations of PI3K activity or protein expression in E1A-expressing versus control cells. Therefore, the contribution of downregulation of Her-2/neu and Axl by E1A to the repression of Akt activation is minimal in these cells that express low levels of Her-2/neu or Axl.

Phosphorylation of protein kinases is tightly regulated by related protein phosphatases, and two phosphatases, PTEN and PP2A, have been shown to repress Akt activation through dephosphorylation.[71,187-201] To determine whether protein phosphatases were involved in the E1A-mediated downregulation of Akt activation, we measured the alteration of protein phosphatases, such as PTEN and PP2A, in stable E1A-expressing cells versus vector control cells. We did not detect any increased expression of PTEN protein in stable E1A-expressing cells versus the controls. Furthermore, there was no change in the expression level of the PP2A regulatory A subunit PP2A/A; however, we did detect elevated expression of the catalytic subunit of PP2A (PP2A/C) in multiple stable E1A-expressing cells (Fig. 3C).[152] We therefore further measured the PP2A activity and found it was enhanced in E1A-expressing MDA-MB-231 cells (231-E1A) in a dose (protein and substrate concentration)-dependent manner compared with that of the vector control cells (231-Vect), suggesting that E1A enhances the activity of PP2A by upregulating PP2A/C expression (Fig. 3D). In addition, we were able to show that activation of PP2A/C is required for E1A–mediated sensitization to drug–induced apoptosis, since blocking PP2A/C expression using a specific small interfering RNA (siRNA) against PP2A/C reduced drug sensitivity in E1A-expressing cells. Deletion mutation of the conserved domain of E1A, which is required for E1A-mediated upregulation of PP2A/C, also abrogated E1A's ability to sensitize cells to drug-induced apoptosis. We also observed that blockade of PP2A activity by a specific PP2A phosphatase inhibitor (okadaic acid) or repression of PP2A/C expression by a specific siRNA resulted in enhanced Akt phosphorylation and reduced p38 phosphorylation, which further suggests that PP2A regulates Akt and p38 activities.[152] In other words, E1A-induced p38 activation resulted from E1A-mediated upregulation of PP2A activity, which in turn repressed Akt activation.

The core enzyme of PP2A is a dimer consisting of a catalytic subunit (PP2A/C) and a regulatory or structural A subunit (PP2A/A). A third regulatory B subunit (PP2A/B), which determines substrate specificity, can be associated with this core structure.[188,190,192,194,196-198,202] The A and C subunit each exist as two isoforms, whereas the 16 B subunits fall into four families. PP2A A subunits are composed of 15 nonidentical tandem repeats of a 39 amino acid sequence, termed a HEAT motif (named after proteins that contain them: huntingtin, elongation factor, A subunit, TOR kinase). The B subunits bind to repeats 1-10, and the C subunits binds to repeats 11-15 of the A subunit. Recent evidence indicates that PP2A forms stable complexes with protein kinase signaling molecules, indicating that it plays a central regulatory role in signal transduction mediated by reversible protein phosphorylation.[188,190,192,194,196-198] Although the role of PP2A in the regulation of apoptosis is not clear, results from a gene

knockout study of PP2A/C imply that PP2A may play a critical role in the regulation of apoptotic signaling.[203] In support of this notion, several groups reported that PP2A, through the dephosphorylation of the key oncogenic survival factor Akt, participated in the regulation of apoptosis induced by ceramide, mistletoe lectin, and 4-hydroxynonenal, an aldehyde product of membrane lipid peroxidation.[204-208] Furthermore, it has been known that PP2A is a Bcl-2 phosphatase and that the PP2A holoenzyme colocalizes with Bcl-2 at the mitochondrial membrane.[209,210] PP2A can be activated by lipid ceramide to dephosphorylate Bcl-2, converting Bcl-2 to a pro-apoptotic molecule. In addition, PP2A can also dephosphorylate Bad and refurbish its pro-apoptotic activity, which is repressed by Akt-mediated phosphorylation and inactivation.[205] Considering the fact that p38 is known to activate PP2A phosphatase activity[211] and that both p38 and Akt can regulate FasL expression and cytochrome *c* release from the mitochondria,[97,174-176,212-215] we reasoned that p38 might also affect Akt activation. To test that possibility, we blocked p38 activation in 231-E1A cells with SB203580, a specific p38 inhibitor. When the level of phosphorylated p38 was inhibited by SB20358, the level of phosphorylated Akt increased (Fig. 4A). Similar results were also observed by using an IPTG-inducible dominant-negative p38 in 231-E1A-expressing cells (Fig. 4A). The results suggested that PP2A/Akt/ASK1/p38 may form a feed-forward loop to regulate cellular response to apoptosis (Fig. 5). To test the notion of this feed-forward regulatory mechanism, we asked whether blockage of Akt activation might also activate PP2A activity. We found that blocking Akt activity by wortmannin indeed increased PP2A activity and induced PARP cleavage, while stimulating Akt activation by growth factor IGF-1 repressed PP2A activity (Fig. 4C). In addition, we also observed that treatment with TNF-α, which has been shown to inhibit Akt phosphorylation, also enhanced PP2A activity and PARP cleavage (Fig. 4D). However, the MEK1/2 kinase inhibitor PD98059 did not affect PP2A activity (Fig. 4D). Blocking Akt activation by using LY249002, another PI3K inhibitor, also increased PP2A activity. Thus, the results support the feed-forward mechanism and E1A, through upregulation of PP2A activity, may turn on this feed-forward mechanism and induce sensitization to apoptosis (Fig. 4D).

This feed-forward loop may represent a highly efficient and coordinated way to drive cells to death. This model is very consistent with all the results presented in this review and the data in the literatures.[37,91,152,154-158,187,199,205,206,211,216,217] For instance, PP2A directly dephosphorylated Akt and blockade of PP2A activity enhanced Akt-phosphorylation and inhibited p38-phosphorylation. Inhibition of Akt activity increased p38-phosphorylation and stimulated PP2A activity, whereas activation of Akt by growth factor IGF-1 repressed PP2A activity. Blocking p38 resulted in elevated Akt phosphorylation. Thus, the feed-forward loop is likely to be a general mechanism for cellular response to apoptosis, as it can be observed in sensitization to apoptosis induced by serum starvation, TNF-α, UV irradiation, and different categories of anticancer drugs. It also suggests that chemotherapeutic drug-induced apoptosis may utilize this feed-forward mechanism by upregulation of p38 and downregualtion of Akt activity and that E1A, by increasing PP2A activity, turns on this feed-forward loop and makes cells more sensitive to a variety of apoptotic stimuli.

Conclusion

The adenoviral E1A-mediated sensitization to anticancer drug-induced apoptosis is a well-established phenomenon in different types of cell systems. However, the molecular mechanisms underlying E1A-mediated chemosensitization are still not yet fully understood. Ever since the first report by Lowe et al[28,40] demonstrates that E1A-mediated activation of p53 is critical for its chemosensitization, the pRb-E2F-ARF-p53-dependent pathway is subsequently delineated. This was further amended by their recent report that an E2F-1-dependent, p53-independent mechanism that controls the transcriptional activation of pro-caspase enzymes also contributes to E1A-mediated chemosensitization in diploid fibroblasts.[130] Mechanisms underlying E1A-mediated chemosensitization in human cancer cells may be more complicated than those observed in normal diploid fibroblasts. For example, downregulation of

Her-2/neu expression is critical for E1A-mediated chemosensitization in Her-2/neu-overexpressing cancer cells, but it may not so critical for E1A-mediated sensitization in diploid normal fibroblast cells, which do not overexpress Her-2/neu. Almost half of human cancers lose functional p53; however, expression of E1A in p53-mutated or functionally inactive cancer cells, such as MDA-MB-231, can also sensitize them to apoptosis induced by different types of anticancer drugs. A similar situation can also apply to ARF in human cancer; in addition, an ARF- and p53-independent mechanism for E1A-mediated chemosensitization is also observed in normal diploid fibroblasts. The apoptotic processes are tightly regulated by both pro- and anti-apoptotic signals, and either external or internal stresses can directly trigger apoptotic cell death program; however, growth factors and growth-stimulating hormones may counteract these death-stimulating stress signals by activation of intracellular cell survival pathways. For example, Akt can block apoptotic processes at different levels through direct phosphorylation and inactivation of Bad (which controls the release of cytochrome *c* from mitochondria) and caspase-9 (which controls downstream caspases activation after cytochrome *c* release from mitochondria). Therefore, it is not surprising for E1A to target such critical intracellular signaling molecules as Akt.

To date, most of domain analysis has pinpointed that the pRB-binding domain of E1A is critical for its sensitization to apoptosis, i.e., ARF and p53 stabilization, downregulation of Akt and upregulation of PP2A and p38, all depend on an intact pRb binding domain, suggesting that disrupting the pRb-E2F-1 pathway is critical in most cases of apoptosis, including those human cancer cells. Therefore, it would be helpful to further defining the roles of pRb and E2F-1 played in E1A-mediated chemosensitization by studying E1A in pRb inactivated human cancer cells or E2F-1-null fibroblast cells.

Understanding the molecular mechanisms underlying E1A-mediated sensitization to anticancer agents can help us to better design future E1A clinical trials and help to select an appropriate patient subpopulation to be recruited into or excluded from the trials. Moreover, results obtained from Phase I/II clinical trials studying combination of E1A gene therapy with chemotherapy may help us to determine whether such regimens may benefit patients who do not respond to conventional chemotherapy.

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HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation

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***HER-2/neu* amplification or overexpression can make cancer cells resistant to apoptosis and promotes their growth. p53 is crucial in regulating cell growth and apoptosis, and is often mutated or deleted in many types of tumour. Moreover, many tumours with a wild-type gene for p53 do not have normal p53 function, suggesting that some oncogenic signals suppress the function of p53. In this study, we show that *HER-2/neu*-mediated resistance to DNA-damaging agents requires the activation of Akt, which enhances MDM2-mediated ubiquitination and degradation of p53. Akt physically associates with MDM2 and phosphorylates it at Ser166 and Ser186. Phosphorylation of MDM2 enhances its nuclear localization and its interaction with p300, and inhibits its interaction with $p19^{ARF}$, thus increasing p53 degradation. Our study indicates that blocking the Akt pathway mediated by *HER-2/neu* would increase the cytotoxic effect of DNA-damaging drugs in tumour cells with wild-type p53.**

The *HER-2/neu* gene (also known as *c-erbB2*) encodes a 185-kDa transmembrane receptor tyrosine kinase that has partial homology with other members of the epidermal growth factor receptor family. Amplification or overexpression of *HER-2/neu* has been found in various cancer types and has been associated with poor clinical outcome, including short survival and short time to relapse[1–3]. We have previously showed that HER-2/*neu* activates the Akt pathway and so confers resistance to tumour necrosis factor (TNF)-induced apoptosis[4], and increases cell proliferation by inducing cytoplasmic localization of $p21^{Cip1/WAF1}$ (ref. 5). The phosphatidylinositol-3 kinase (PI(3)K)–Akt pathway plays an important role in preventing cells from undergoing apoptosis and contributes to the pathogenesis of malignancy[6,7]. Several targets of the PI(3)K–Akt signalling pathway have recently been identified that might underlie the ability of this regulatory cascade to promote cell survival and cell growth. These substrates include two components of the intrinsic cell death machinery (Bad and caspase 9; refs 8,9), transcription factors of the forkhead family[10,11], Ikk-α (a kinase that regulates NF-κB activation[12]) and $p21^{Cip1/WAF1}$ (a cell-cycle inhibitor that controls cell growth[5]). The PI(3)K–Akt pathway has also been reported to delay p53-mediated apoptosis[13], suggesting that there is a link between the PI(3)K–Akt signalling pathway and p53-mediated apoptosis.

The tumour-suppresser protein p53 is a transcription factor that can induce either growth arrest or apoptosis and is frequently mutated or deleted in many types of tumour[14–21]. In addition, many tumours with a wild-type gene for p53 do not have functional p53 protein, suggesting that some oncogenic signals suppress the function of p53 (refs 14,17). The levels and activity of p53 are controlled largely by MDM2, which is amplified or overexpressed in a variety of human tumours and can function as an oncogene in tissue culture systems[14,17,20]. MDM2 can bind directly to p53 and promote its ubiquitination and subsequently its degradation by the proteasome[20,21]. The ability of MDM2 to degrade p53 depends on its ubiquitin E3 ligase activity and its nuclear localization signal (NLS) and nuclear export signal (NES), which are required for MDM2 to shuttle between the nucleus and the cytoplasm[14,20]. The nucleus–cytoplasm shuttling of MDM2 presumably mediates p53 degradation by cytoplasmic proteasomes.

In addition to MDM2, the transcriptional co-activator CBP/p300 has also been shown to play a role in efficient degradation of p53 (refs 22,23). CBP/p300 forms a complex with MDM2 *in vitro* and *in vivo*, and provides a platform to allow the assembly of the protein complex necessary for MDM2-mediated ubiquitination and degradation of p53 (ref. 23). MDM2 is feedback regulated by p53 and by the expression of ARF protein (p14 in humans and p19 in mice), which is encoded by the *Ink4A* locus[15,24–26]. Loss of ARF increases tumour susceptibility in mice, and mutations in *Ink4A* are often detected in human cancers[26]. The ARF protein binds directly to MDM2 to block p53 degradation by inhibiting the ubiquitin E3 ligase activity associated with MDM2 (ref. 27) and by sequestering MDM2 in nucleoli to prevent its export to the cytoplasm[28,29]. Interestingly, the regions that control MDM2 nucleus–cytoplasm shuttling (amino acids 181–185 of the NLS and amino acids 191–205 of the NES)[20] and the regions required for p300 binding (amino acids 102–222)[22,23] and $p19^{ARF}$ binding (amino acids 154–221)[24] overlap, suggesting that p300, $p19^{ARF}$ and other cellular proteins interact to control the function of MDM2.

In this study, we show that *HER-2/neu*-mediated resistance to DNA-damaging agents requires the activation of Akt, which enhances MDM2-mediated ubiquitination and degradation of p53. Akt interacts physically with MDM2 and phosphorylates it at Ser166 and Ser186, and so increases the nuclear localization of MDM2. Furthermore, phosphorylation of MDM2 increases its interaction with p300 and inhibits its interaction with $p19^{ARF}$ and so increases p53 degradation. Our study indicates that blocking the Akt pathway mediated by *HER-2/neu* would increase the cytotoxic effect of DNA-damaging drugs in tumour cells with wild-type p53.

Results

Blocking the Akt pathway sensitizes *HER-2/neu*-transformed cells to DNA-damaging agents. Because overexpression of *HER-2/neu* induces resistance to apoptosis mediated by chemotherapeutic drugs[3,4,30,31] and the Akt pathway is known to increase cell survival, we examined whether blocking the Akt pathway would sensitize cells to DNA-damaging drugs in our model system. This system

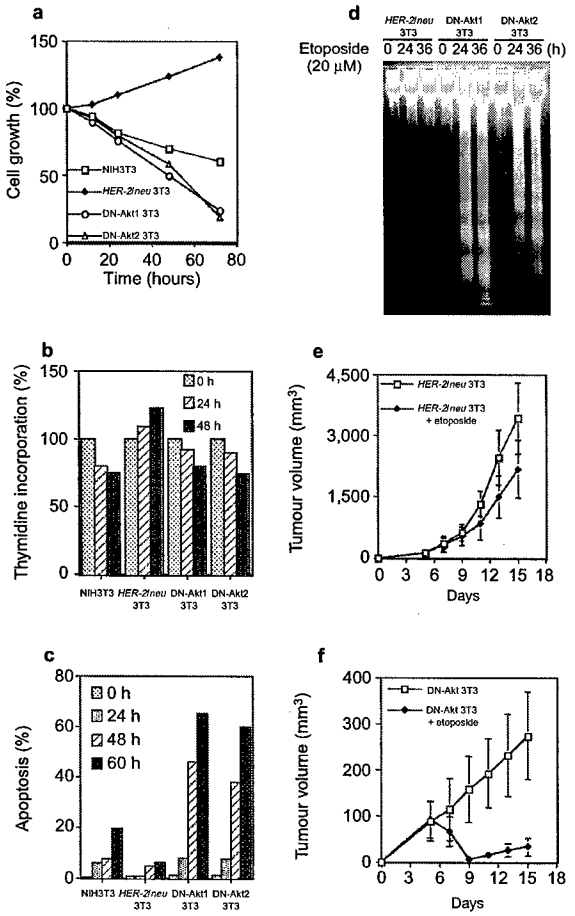

Figure 1 **The Akt pathway is required for *HER-2/neu*-mediated chemoresistance to etoposide. a**, Blocking the Akt pathway reduces the growth of DN-Akt 3T3 cells in the presence of etoposide. The cells (3×10^3) were seeded in 96-well plates and grown in Dulbecco's modified Eagle's medium/F12 medium plus 5% foetal bovine serum in presence of 20 μM etoposide. The growth rate was monitored by the MTT assay. The results are presented as the mean ± s.e.m. of three independent experiments performed in quadruplicate. **b**, Cells (3×10^3) were grown as described in (**a**) with 1 μCi of [^{3}H]-thymidine for 12 h. The cell replication rate was determined by measuring [^{3}H]-thymidine incorporation. The results are presented as the mean ± s.e.m. of three independent experiments performed in quadruplicate. **c**, Blocking the Akt pathway significantly induced apoptosis in DN-Akt 3T3 cells. The cells were grown in 5% serum plus 20 μM etoposide, and the percentage of cells in apoptosis was measured by fluorescence-activated cell sorting as described previously[4]. The results are presented as the mean ± s.e.m. of three independent experiments. **d**, Blocking the Akt pathway induced etoposide-mediated apoptosis in DN-Akt 3T3 cells, as measured by the DNA fragmentation assay[4]. The cells were grown in 100-mm plates as described in (**a**) with 20 μM etoposide. **e**, **f**, Tumours induced by DN-Akt 3T3 cells were much more sensitive to etoposide treatment. *HER-2/neu* 3T3 cells (**e**) and DN-Akt 3T3 cells (**f**) were inoculated subcutaneously in nude mice. One group of five mice from each cell line was treated with intravenous injections of etoposide on days 5 and 7 after the inoculation (solid symbols). The control groups (open symbols) were injected with the same volume of saline. The tumour volume was measured every two days, and the data represent the mean ± s.d. from five mice.

consists of NIH3T3 cells, *HER-2/neu* 3T3 cells (*HER-2/neu*-transformed NIH3T3 cells) and DN-Akt 3T3 cells (*HER-2/neu* 3T3 cells transfected with *DN-Akt*, a mutant *Akt* gene that produces a protein with no kinase activity)[4]. As expected, the *HER-2/neu* 3T3 cells were not affected by the DNA-damaging agent etoposide (Fig. 1). The Akt pathway is known to be constitutively activated in *HER-2/neu* 3T3 cells[4] and, when this pathway was blocked by DN-Akt in DN-Akt 3T3 cells, the cells grew much more slowly (Fig. 1a). When the DNA synthesis rate was determined by measuring [^{3}H]-thymidine incorporation, *HER-2/neu* 3T3 cells did not synthesize more DNA than the parental NIH3T3 cells or DN-Akt 3T3 cells (Fig. 1b).

The net cell growth rate depends on a fine balance between the cell proliferation rate and the cell death rate, and so we next examined whether apoptosis contributes to the difference in growth in these cells. There was significant difference in apoptosis among these cells as measured by fluorescence-activated cell sorting (FACS) analysis (Fig. 1c). The DN-Akt 3T3 cells underwent more apoptosis than did the *HER-2/neu* 3T3 cells after treatment with etoposide. The apoptosis in these cells was further confirmed by the DNA fragmentation in these cells (Fig. 1d). Therefore, the reduction in cell growth in DN-Akt 3T3 cells after treatment with etoposide was probably due to the increased apoptosis in these cells. Similarly, we treated these cells with another DNA-damaging agent, doxorubicin, and found that DN-Akt 3T3 cells were also more sensitive to apoptosis than *HER-2/neu* 3T3 cells (data not shown).

To examine whether the phenomenon can be observed *in vivo*, we inoculated *HER-2/neu* 3T3 cells and DN-Akt 3T3 cells into nude mice to generate mammary tumours, and tested the susceptibility of these tumours to DNA-damaging agents. Tumours from *HER-2/neu* 3T3 cells were not sensitive to etoposide (Fig. 1e), supporting the notion that *HER-2/neu*-overexpressing tumours are resistant to chemotherapeutic drugs, including DNA-damaging agents[3,30]. However, when the Akt pathway was blocked by DN-Akt, the tumours induced by DN-Akt 3T3 cells became very sensitive to etoposide (Fig. 1f). The dramatic tumour shrinkage after etoposide treatment is probably due to sensitization to apoptosis because massive apoptosis in these tumours was detected by the TUNEL assay (data not shown). Together, our results indicate that the Akt pathway is required for *HER-2/neu*-mediated drug resistance to DNA-damaging agents. Blocking this pathway sensitized *HER-2/neu*-overexpressing cells to DNA-damaging agents both *in vitro* and *in vivo*.

Activation of Akt-induced p53 ubiquitination and degradation. Because etoposide inhibits DNA topoisomerase II and causes DNA breaks, and because p53 is a sensor for damaged DNA and is induced by DNA damage[21,32], we tested whether p53 is involved in this sensitization effect mediated by the blockage of Akt pathway. We treated two pairs of cell lines ($p53^{-/-}$ and wild-type MEF cells, and p53-mutated MDA-MB453 and its DN-Akt/MDA453 transfectants) with etoposide. The p53-wild-type MEF cells were very sensitive to etoposide treatment (Fig. 2a). Blocking the Akt pathway using Wortmannin alone did not affect cell survival, but it dramatically enhanced the sensitization to etoposide treatment in p53-wild-type MEF cells (Fig. 2a). However, no effect was observed in the $p53^{-/-}$ cells (Fig. 2a). Similarly, blocking the Akt pathway with DN-Akt in p53-mutated DN-Akt/MDA453 cells could not sensitize the cells to etoposide treatment (Fig. 2b), although they did respond to TNF-induced apoptosis, as shown previously[4]. When similar experiments were performed in $p53^{-/-}$ and p53-wild-type cells, and apoptosis was measured by FACS analysis, we observed the same results (data not shown). Thus, these results suggest that p53 is crucial for etoposide-induced apoptosis.

To examine whether blockage of the Akt pathway would further sensitize p53-wild-type cells to etoposide-induced apoptosis in other cell types, we treated another two p53-wild-type (HBL-100 and MCF-7) and two p53-mutated (MDA-MB231 and SKOV3-ip1) cancer cell lines with etoposide. We found that the two p53-wild-type cell lines (HBL-100 and MCF-7) were more sensitive to apoptosis (as measured by FACS analysis) when the Akt pathway was blocked by a PI(3)K inhibitor (LY294002). However, in the two p53-mutated cells, the PI(3)K inhibitor could not sensitize the cells

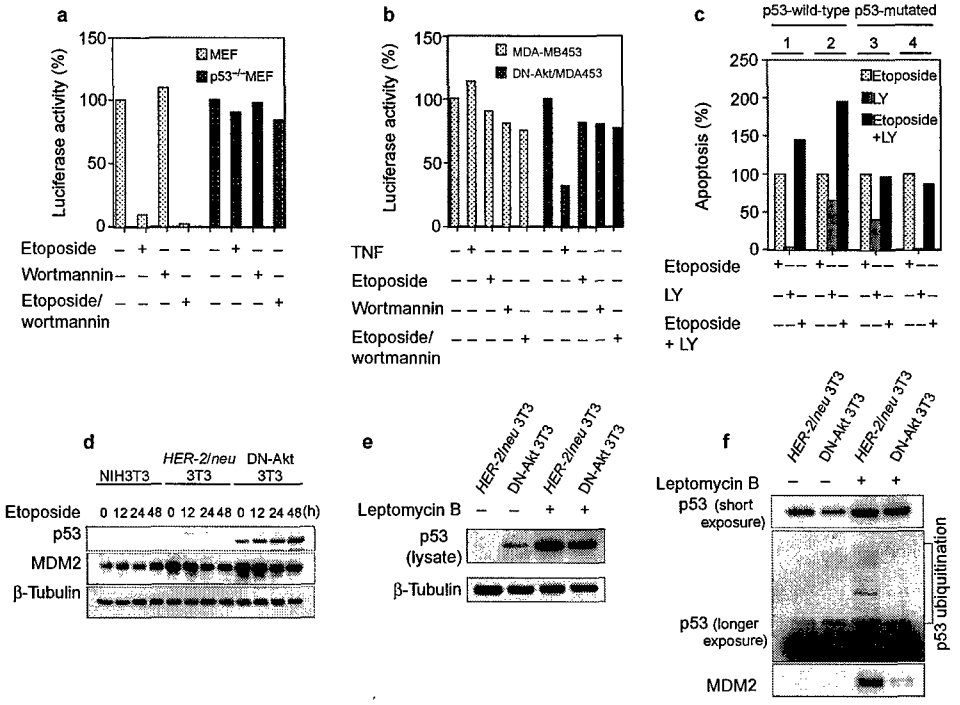

Figure 2 **Activation of Akt induced p53 ubiquitination and degradation.** **a**, The presence of p53 protein was required for sensitization to etoposide treatment. The p53-wild-type MEF and $p53^{-/-}$ MEF cells were transfected with 2 μg of a plasmid that contains actin-promoter-driven luciferase. After 12 h of transfection, cells were treated with 20 μM etoposide, 100 nM Wortmannin or 100 nM of Wortmannin plus 20 μM of etoposide and incubated for another 36 h. The cell lysates were prepared and the luciferase activity in each sample was measured. The sensitization to etoposide treatment was presented by the luciferase activity (mean ± s.e.m. in two separate experiments) over the control without any treatment. **b**, Blockage of the Akt pathway did not sensitize p53-mutated MDA-MB453 cells to etoposide treatment. Breast cancer cell line MDA-MB453 and its DN-Akt transfectants (DN-Akt/MDA453) cells were transfected with 2 μg of a plasmid that contains actin-promoter-driven luciferase. After 12 h of transfection, the cells were treated with 40 ng ml^{-1} of tumour necrosis factor, 20 μM etoposide, 100 nM of Wortmannin or 100 nM of Wortmannin plus 20 μM of etoposide as described in (**a**). The cell lysates were prepared and luciferase activity in each sample was measured. The sensitization to etoposide treatment was presented by the luciferase activity (mean ± s.e.m. in two separate experiments) over the control without any treatment. **c**, Blocking the Akt pathway sensitized p53-wild-type but not p53-mutated cells to etoposide-induced apoptosis. Two p53-wild-type (HBL-100 and MCF-7) and two p53-mutated (MDA-MB231 and SKOV3-ip1) cancer cell lines were treated with 20 μM etoposide in the presence or absence of the PI(3)K inhibitors LY294002 (50 μM) and Wortmannin (0.1 μM) (LY). Apoptotic cells in each sample were measured by flow cytometry using a fluorescence-activated cell sorter with propidium iodide staining. The two p53-wild-type cell lines were treated with etoposide and/or PI(3)K inhibitors for 24 h and harvested for analysis. Because few cells were found undergoing apoptosis in 24 h for the two p53-mutated cell lines, these two lines were treated with etoposide for 48 h to observe the apoptotic effect mediated by VP16. Apoptotic cells treated with etoposide alone in each sample were defined as 100%. Cell lines 1–4 were HBL-100, MCF-7, MDA-MB231 and SKOV3-ip1, respectively. **d**, Blockage of the Akt pathway led to production of p53. NIH3T3, *HER-2/neu* 3T3 and DN-Akt 3T3 cells were treated with 20 μM etoposide, for the indicated times. Whole cell lysates (50 μg) were prepared and subjected to western blot analyses with antibodies specific for p53 (Ab-7), MDM2 and β-tubulin. **e**, Blocking nuclear export induced p53 stabilization in *HER-2/neu* 3T3 and DN-Akt 3T3 cells. *HER-2/neu* 3T3 and DN-Akt 3T3 cells were treated with 5 ng ml^{-1} leptomycin B for 7 h or not treated, and the p53 and β-tubulin levels were analysed by western blotting. **f**, Activation of Akt induced the ubiquitination of p53 and increased the binding of MDM2 to p53. *HER-2/neu* 3T3 and DN-Akt 3T3 cells were treated with leptomycin B as in (**e**) or left alone. The cell lysates (1.2 mg of *HER-2/neu* 3T3 protein and 0.4 mg of DN-Akt 3T3 protein) were immunoprecipitated with monoclonal anti-p53 antibody (Ab-1) and the precipitates were then subjected to western blotting with polyclonal anti-p53 antibody (Ab-7) for a short (10 sec) or a longer (1 min) exposure. The same membrane was used again for western blotting against MDM2.

to etoposide-induced apoptosis (Fig. 2c). Similar results were also obtained when we used another PI(3)K inhibitor, Wortmannin (data not shown). Therefore, sensitization to etoposide-induced apoptosis occurred only in p53-wild-type cells, not in p53-mutated or $p53^{-/-}$ cells, suggesting the involvement of p53 and Akt in the etoposide-induced apoptosis.

We next examined the expression of p53 in NIH3T3, *HER-2/neu* 3T3 and DN-Akt 3T3 cells before and after treatment with etoposide. We did not observe the induction of p53 expression in NIH3T3 and *HER-2/neu* 3T3 cells, but did find time-dependent induction of p53 in DN-Akt 3T3 cells (Fig. 2d). Surprisingly, basal expression of p53 in DN-Akt 3T3 cells was dramatically elevated (Fig. 2d), indicating that blocking the Akt pathway increases the p53 protein level. The half-life of p53 is ~15 min, and p53 is present at low levels in cells and only increases in response to physiological stress such as DNA damage[21,32]. The p53 protein level increases as a result of post-translational modification, which stabilizes the protein and is largely controlled by MDM2. MDM2 can bind to p53, promote its ubiquitination and shuttle it to cytoplasm, where it is subsequently degraded by the proteasome. Leptomycin B (LMB), a cytotoxin produced by streptomycetes, can block nuclear export and stabilize p53 accumulated in the nucleus of LMB-treated cells[33]. To rule out the possibility that the lack of expression of p53 in *HER-2/neu* 3T3 cells was due to a genetic defect in these cells, we treated the cells with LMB. The concentration of p53 increased in *HER-2/neu* 3T3 cells after treatment with

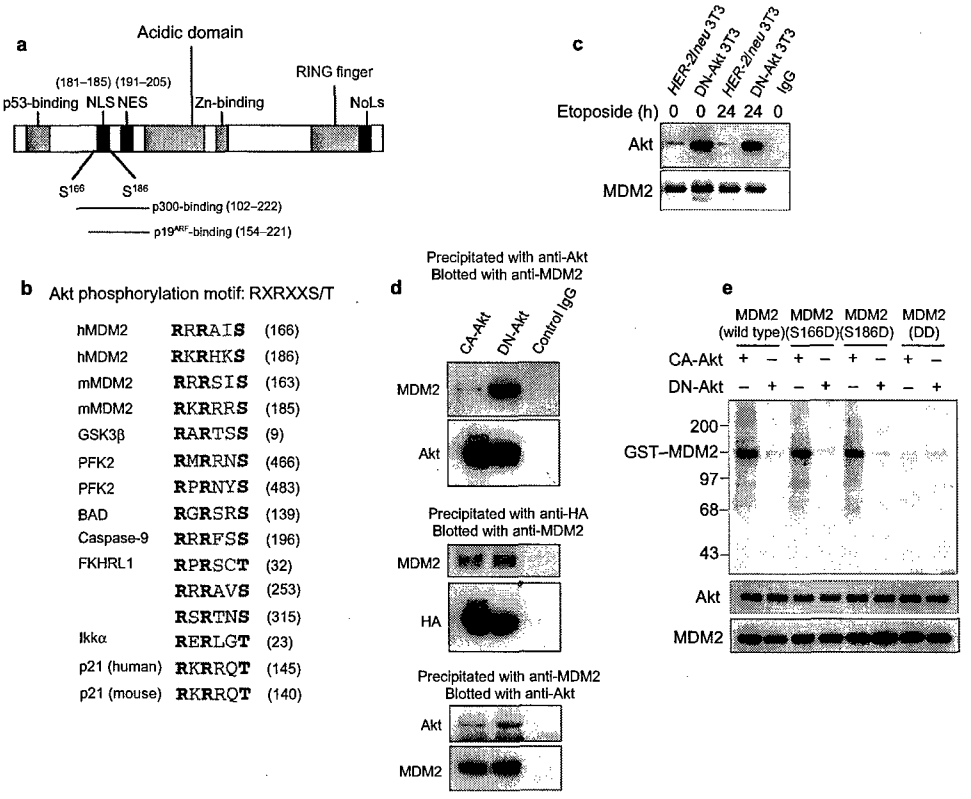

Figure 3 **Akt interacts with MDM2 and phosphorylates Ser166 and Ser186.** **a**, Structure of the MDM2 protein, showing the positions of the nuclear-localization signal (NLS), the nuclear-export signal (NES) and domains that interact with p300 and $p19^{ARF}$. **b**, The consensus Akt phosphorylation motif is highlighted. Sequences of MDM2 and other known Akt substrates are shown for comparison. **c**, Immunoprecipitation of endogenous MDM2 and detection of endogenous Akt. Endogenous MDM2 was immunoprecipitated from 1000 μg of *HER-2* 3T3 or DN-Akt 3T3 cell lysates with an MDM2-specific antibody (SMP14) or control IgG. After transfer to a nitrocellulose membrane, endogenous Akt was detected with an Akt-specific antibody (Upstate Biotechnology). **d**, Association between Akt and MDM2. HA-tagged DN-Akt or CA-Akt (10 μg) was transfected into 293T cells by the calcium phosphate method. The cells were lysed in RIPA buffer after 48 h and Akt was immunoprecipitated with anti-Akt (Upstate Biotechnology) or ani-HA (HA.11; Babco) antibody. After transfer to a nitrocellulose membrane, MDM2 was detected with MDM2-specific antibody. Alternatively, the cell lysates were immunoprecipitated with MDM2-specific antibody. After transfer to a nitrocellulose membrane, the bound Akt was detected with Akt-specific antibody. **e**, Akt phosphorylated MDM2 at Ser166 and Ser186. HA-tagged CA-Akt or DN-Akt (20 μg) was transiently transfected into 293T cells as described in Methods. After 48 h of incubation, CA-Akt and DN-Akt were immunopricipitated with an anti-HA antibody and incubated with 5 μg of either GST–wild-type MDM2 or GST–mutant MDM2 (S166D, S186D and S166,186DD) in a kinase buffer containing 5 μCi of [γ^{32}P]ATP for 30 min at 30 °C. The kinase reaction was stopped by the addition of SDS-PAGE buffer and the samples were assayed by autoradiography. The lower two panels show western blots for Akt and GST–MDM2 used in the phosphorylation reaction, detected with antibodies against Akt and MDM2, respectively.

LMB (Fig. 2e). These results indicate that the absence of p53 in *HER-2/neu* 3T3 cells was not caused by a genetic defect but rather by the destabilization of p53 in these cells.

To compare the ubiquitination of p53 in these cells, we used more cell lysate from *HER-2/neu* 3T3 cells than from DN-Akt 3T3 cells to produce equal amounts of immunoprecipitated p53 before and after treatment with LMB. Surprisingly, there was dramatically more ubiquitination of p53 in *HER-2/neu* 3T3 cells than in DN-Akt 3T3 cells (Fig. 2f). The difference in ubiquitination of p53 was not due to a difference in p53 levels, because nearly equal amounts of p53 were immunoprecipitated from *HER-2/neu* 3T3 and DN-Akt 3T3 cells (short exposure in Fig. 2f). When the same membrane was analysed by western blot using MDM2-specific antibody, we found more MDM2 binding to p53 immunoprecipitated from *HER-2/neu* 3T3 cells (Fig. 2f). Thus, the increased ubiquitination of p53 observed in *HER-2/neu* 3T3 cells resulted largely from increased binding of MDM2 to p53 in these cells (Fig. 2f). These results indicate that the activation of Akt could increase the binding of MDM2 to p53 and induce the ubiquitination of p53. Blocking the Akt pathway stabilized the p53 protein by inhibiting the binding of MDM2 to p53, consequently suppressing the ubiquitination and degradation of p53.

Akt interacts with and phosphorylates MDM2. The inability of MDM2 to bind and ubiquitinate p53 in DN-Akt 3T3 cells seems to be responsible for the cell's increased p53 level and sensitization to DNA-damaging agents. Recently, MDM2 was found to be phosphorylated by ATM (for ataxia-telangiectasia mutated), disrupting its binding to p53 and leading to the stabilization of p53 (ref. 34). We noticed that there were two potential Akt phosphorylation sites (Ser166 and Ser186) in the MDM2 protein (Fig. 3a), and that these two sites were highly conserved across species (Fig. 3b). These two potential phosphorylation sites were in the regions crucial for MDM2 nuclear import and export, and required for p300 and $p19^{ARF}$ binding. To determine whether Akt interacts with and phosphorylates MDM2, and so regulates its function, we performed immunoprecipitation experiments to detect the association

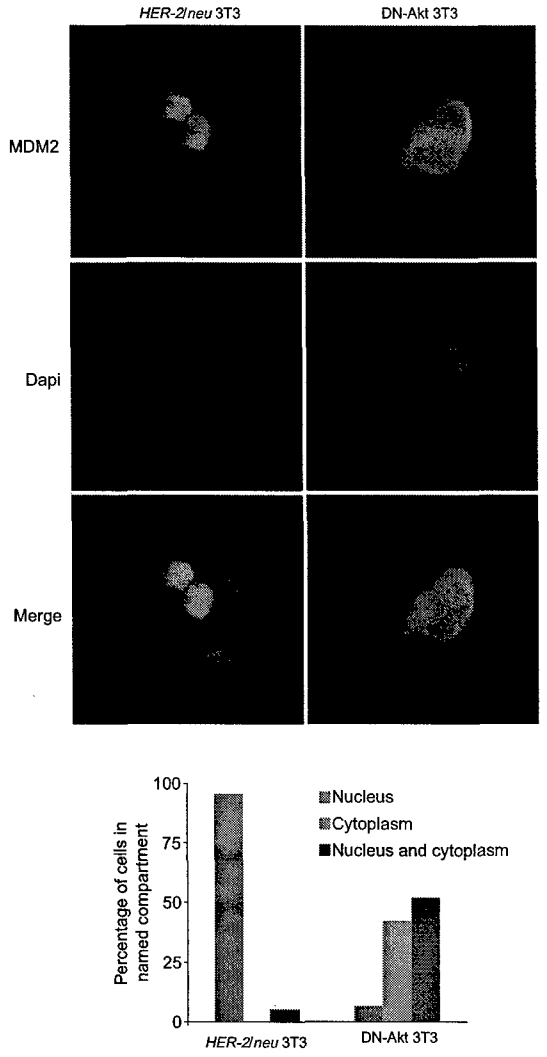

Figure 4 **Akt affects the cellular localization of endogenous MDM2.** **a**, ~2×10⁴ *HER-2* 3T3 and DN-Akt 3T3 cells were plated into chamber slides for 16 h. After fixation of the samples in 4% paraformaldehyde for 1 h and permeabilization with 0.2% Triton X-100 for 30 min, the cellular localization of MDM2 was detected using a monoclonal antibody against MDM2 (SMP14). After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG plus DAPI and examined under a fluorescent microscope (Zeiss). **b**, Quantification of subcellular MDM2 staining. The subcellular localizations of MDM2 in *HER-2* 3T3 and DN-Akt 3T3 cells were assessed in 100 cells in several different views. The graph shows the percentages of cells with the indicated MDM2 subcellular localization.

between Akt and MDM2. In an immunoprecipitation of endogenous MDM2, we detected the presence of endogenous Akt (Fig. 3c). The binding of Akt to MDM2 was increased in DN-Akt 3T3 cells (Fig. 3c). Treatment with etoposide does not seem to affect the interaction between MDM2 and Akt. We also transfected 293T cells with haemagglutinin (HA)-tagged constitutively active Akt (CA-Akt) or DN-Akt. After immunoprecipitating Akt or HA, we detected MDM2, and vice versa (Fig. 3d), indicating that these two molecules are associated. MDM2 seems to associate more strongly with DN-Akt than with CA-Akt.

To examine whether Akt can phosphorylate MDM2, we transfected CA-Akt and DN-Akt into 293T cells and immunoprecipitated Akt from cell lysates. After incubation with glutathione-*S*-transferase (GST)–MDM2 (wild-type), GST–MDM2 (S166D), GST–MDM2 (S186D) or GST–MDM2 (S166, 186DD) in a kinase reaction mixture, we found that Akt phosphorylated MDM2 at Ser166 and Ser186 with equal efficiency, whereas DN-Akt could not (Fig. 3e). When both Ser166 and Ser186 were mutated to aspartic acid, CA-Akt could not phosphorylate this double mutant of MDM2, suggesting that Ser166 and Ser186 in MDM2 were specific for Akt phosphorylation (Fig. 3e). The fact that no phosphorylation was observed in the double mutant of MDM2 (S166,186DD) was not due to the lack of Akt kinase or MDM2 proteins, because nearly equal amount of kinase and protein were used (Fig. 3e, bottom two panels). These results indicate that Akt physically interacted with MDM2 and phosphorylated MDM2 at Ser166 and Ser186.

Akt induces nuclear localization of MDM2. Because the Akt phosphorylation sites in MDM2 are close to the NLS and NES, we examined whether the activation of Akt affects the cellular localization of MDM2. We performed immunofluorescence staining to analyse the cellular localization of endogenous MDM2 in *HER-2/neu* 3T3 and DN-Akt 3T3 cells. In the parental *HER-2/neu* 3T3 cells, MDM2 was usually found in the nucleus (95% of cells), with little in the cytoplasm (Fig. 4a). However, in DN-Akt-transfected cells, MDM2 was either in the cytoplasm (42%) or distributed evenly between the cytoplasm and nucleus (50%) (Fig. 4b). MDM2 has been previously reported to localize mainly to the nucleus. The inability of MDM2 to localize to the nucleus in DN-Akt 3T3 cells suggests that phosphorylation of MDM2 by Akt is required for its nuclear translocation. Blocking the Akt pathway in DN-Akt 3T3 cells would inhibit the nuclear import of MDM2, and so result in the stabilization of p53.

We next tested whether the phosphorylation of MDM2 by Akt affects the cellular localization of MDM2. We transfected $p53^{-/-}$ and $MDM2^{-/-}$ MEF cells with CA-Akt or DN-Akt together with wild-type or mutant MDM2 and examined the cellular localization of MDM2 by immunofluorescence analysis. As shown in Fig. 5, wild-type MDM2 was found predominantly in the nucleus in the presence of CA-Akt but was localized to both the cytoplasm and the nucleus when DN-Akt was introduced. Mutants of MDM2 (S166D, S186D or S166,186DD, in which serine 166 or/and serine 186 were replaced with aspartic acid to mimic the phosphorylation of MDM2 by Akt) were found predominantly in the nucleus even in the presence of DN-Akt. These results strongly indicate that Ser166 and Ser186 of MDM2 are crucial in determining the cellular localization of MDM2, and that the phosphorylation of Ser166 and Ser186 of MDM2 by Akt resulted in the nuclear localization of MDM2. Taken together, our results indicate that Akt can interact with MDM2 and phosphorylate the Ser166 and Ser186 residues in MDM2, leading to the nuclear localization of MDM2.

These results establish the regulation of MDM2 by the HER-2/*neu*–Akt pathway in cell culture. To examine whether this phenomenon also existed in tumour tissues, we compared the levels of activated (phosphorylated) Akt and the cellular localization of MDM2 in 33 cases of human breast tumours that are either HER-2/*neu* and Akt positive or HER-2/*neu* and Akt negative by immunostaining. In 21 HER-2/*neu* and Akt positive breast tumour tissues, we found that MDM2 was mainly present in the nucleus. By contrast, in all 12 HER-2/*neu* and Akt negative breast tumour tissues examined, MDM2 was localized in both the nucleus and cytoplasm (Fig. 6). Similar results were obtained when we used another MDM2 antibody (NCL-MDM2; Novocastra Laboratories, Newcastle upon Tyne, UK) (data not shown). The subcellular localization of MDM2 and the activation of Akt were significantly correlated based on the analysis using Fisher's exact test (Table 1). Thus, the correlation between these two molecules in primary tumour samples was consistent with our *in vitro* data from cell lines and transfection studies (Figs 4, 5). The tumour staining data agree

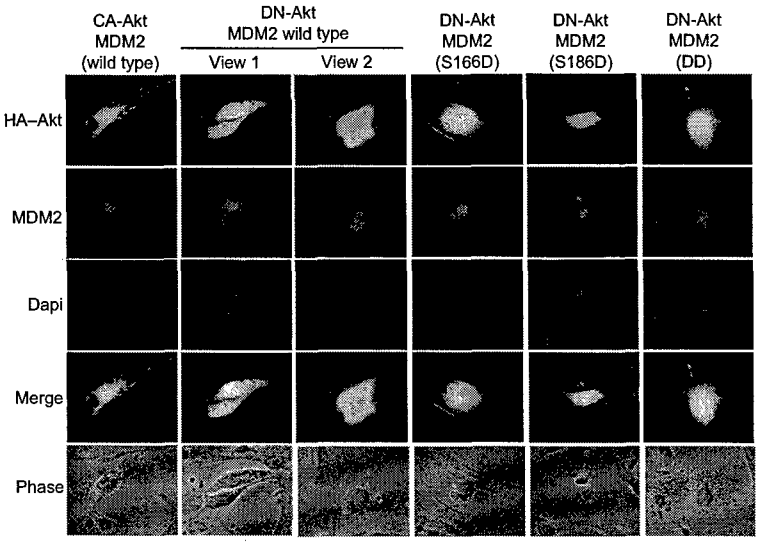

Figure 5 **Akt affects the subcellular localization of MDM2.** A 1:1 ratio of CA-Akt or DN-Akt (2 μg) and the wild-type or mutant MDM2 (2 μg) were transfected into $p53^{-/-}$ and $MDM2^{-/-}$ MEF cells. After 36 h of incubation, the cells were trypsinized and plated into chamber slides for another 12 h. After fixation, the cellular locations of CA-Akt, DN-Akt and MDM2 were detected using antibodies against HA (HA.11, Babco) and MDM2 (Ab-2, Oncogene Research). After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG plus Dapi, and examined under a fluorescent microscope (Zeiss). When wild-type MDM2 was transfected with CA-Akt, MDM2 were found in the nucleus in ~95% of the transfected cells. However, when MDM2 was transfected with DN-Akt, 25% of the transfected cells had MDM2 staining only in the nucleus and 75% of the cells had MDM2 staining in both the nucleus and the cytoplasm. When the S166D, S186D or S166,186DD mutant of MDM2 was transfected with DN-Akt, ~90% of the cells had MDM2 staining only in the nucleus.

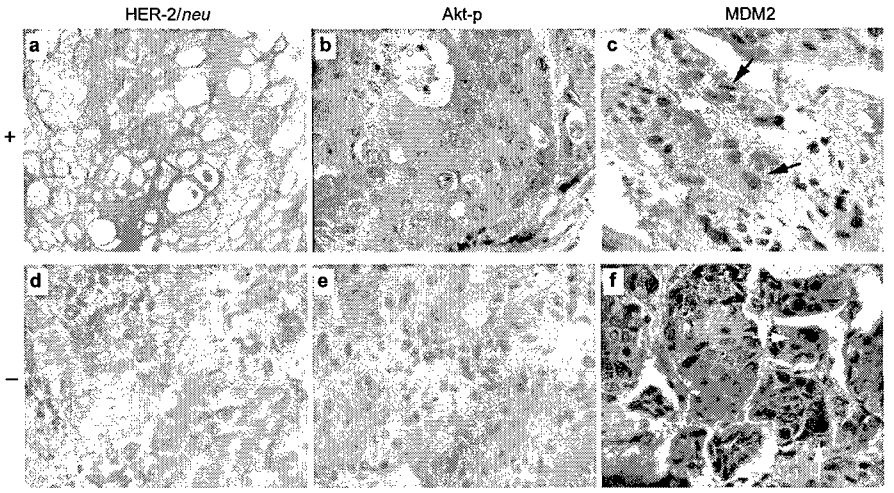

Figure 6 **HER-2/*neu* activates Akt and induces nuclear localization of MDM2 in breast tumour tissues.** Tissue sections from the HER-2/*neu*- and Akt-positive adenocarcinomas (**a–c**) and HER-2/*neu*- and Akt-negative adenocarcinomas (**d–f**) were stained with antibodies specific to HER-2/*neu* (**a** and **d**), phosphorylated Akt (**b** and **e**), MDM2 (**c** and **f**) or normal rabbit serum (data not shown). The immunostaining was visualized with peroxidase-conjugated secondary antibody. The arrows indicate the nuclear and cytoplasmic localization of MDM2 in **c** and **f**, respectively.

with the observation in cell culture and further strengthen the hypothesis that overexpression of *HER-2/neu* can regulate the cellular distribution of MDM2 via the activation of Akt.

Phosphorylated MDM2 induces preferential binding to p300 but not to $p19^{ARF}$. Because the Akt phosphorylation sites Ser166 and Ser186 in MDM2 were also within the region required for MDM2 to bind p300 and $p19^{ARF}$, we investigated whether the loss of p53 ubiquitination in DN-Akt 3T3 cells was due to changed binding of MDM2 to p300 and $p19^{ARF}$. We immunoprecipitated MDM2 from *HER-2/neu* 3T3 or DN-Akt 3T3 cells and examined

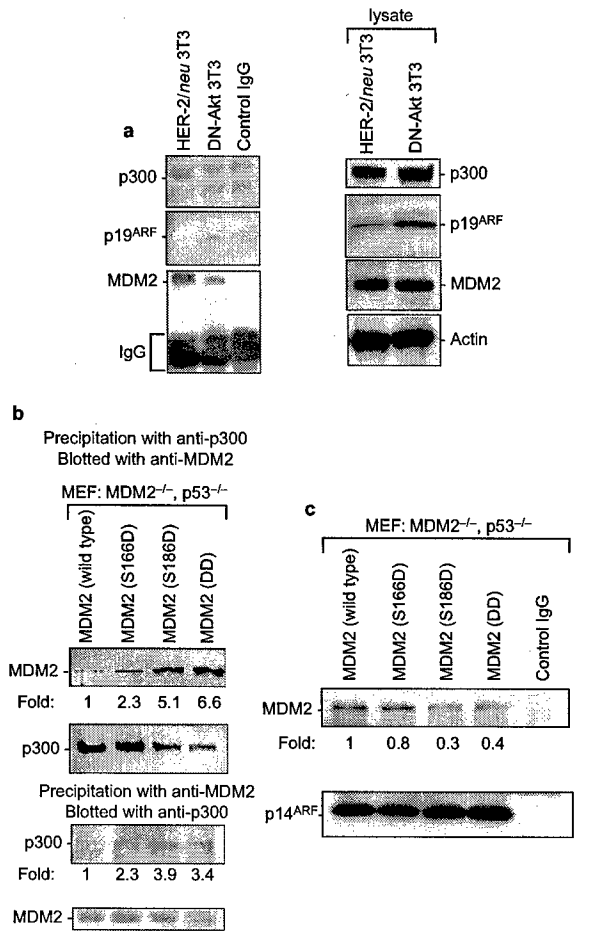

Figure 7 **Phosphorylated MDM2 binds differently to p300 and p19^{ARF}.** **a**, MDM2 from *HER-2/neu* 3T3 and DN-Akt 3T3 cells had different binding affinities to p300 and p19^{ARF}. Cell lysates from *HER-2/neu* 3T3 or DN-Akt 3T3 cells were immunoprecipitated with monoclonal anti-MDM2 antibody (SMP14) and the precipitates were then subjected to western blotting analysis for the binding of p300 and p19^{ARF}. The same lysates were used for western blotting analysis for the expression of p300, p19^{ARF}, MDM2 and actin. **b**, Preferential binding of the phosphorylated form of MDM2 to p300. The wild-type and phosphorylated forms of MDM2 mutants were transfected into p53$^{-/-}$ and MDM2$^{-/-}$ cells. After 48 h of culture, the cell lysates were immunoprecipitated with anti-p300 and anti-MDM2 antibodies, and the precipitates were subjected to western blot analysis for the binding of MDM2 and p300. The intensities of the bands were quantified by densitometry using NIHimage and are shown under the western blots. **c**, Weaker binding of phosphorylated form of MDM2 to p14^{ARF}. The wild-type and phosphorylated forms of MDM2 were transfected with p14^{ARF} into p53$^{-/-}$ and MDM2$^{-/-}$ cells. After 48 h of culture, the cell lysates were immunoprecipitated with antibodies against p14^{ARF} and the precipitates were subjected to western blot analysis for the binding of MDM2. The intensity of the bands was quantified by densitometry using NIHimage and are shown under the western blot.

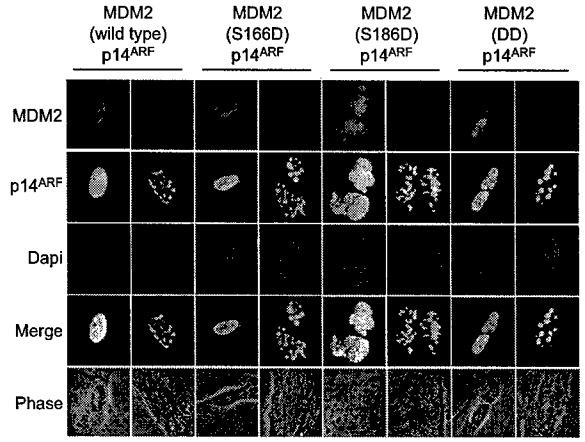

Figure 8 **Nuclear localization of wild-type and mutant MDM2 is not affected by the presence of p14^{ARF}.** A 1:1 ratio of wild-type or mutant MDM2 (2 μg) and p14^{ARF} (2 μg) were transfected into p53$^{-/-}$ and MDM2$^{-/-}$ MEF cells. After 36 h of incubation, the cells were trypsinized and plated into chamber slides for another 12 h. After fixation, the cellular localization of MDM2 and p14^{ARF} were detected by using antibodies against MDM2 (SMP14) and p14^{ARF} (Ab-1; Neomarkers, Fremont, California), respectively. After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit plus Dapi and examined under a fluorescent microscope (Zeiss). For each transfection staining, cells producing both MDM2 and p14^{ARF} are shown on the left-hand side and cells expressed only p14^{ARF} are shown on the right-hand side.

MDM2's association with p300 and p19^{ARF}. We found that MDM2 from *HER-2/neu* 3T3 cells bound much more strongly to p300 than in DN-Akt 3T3 cells (Fig. 7a). However, p19^{ARF} binding to MDM2 was dramatically lower in *HER-2/neu* 3T3 cells than in DN-Akt 3T3 cells (Fig. 7a). The differences in p300 and p19^{ARF} bound by MDM2 between *HER-2/neu* 3T3 and DN-Akt 3T3 cells were not due to a difference in protein levels, because equal amounts of MDM2 were immunoprecipitated from these cells and the expression of p300 and p19^{ARF} in these cells was the same (Fig. 7A). We also transfected wild-type MDM2 and phosphorylated forms of MDM2 (S166D, S186D or S166,186DD) into MDM2$^{-/-}$ and p53$^{-/-}$ cells, and analysed the binding of these phosphorylated forms of MDM2 with p300. As seen in Fig. 7b, after immunoprecipitating the p300, the association of phosphorylated forms of MDM2 to p300 was stronger than that of wild-type MDM2. Similarly, when we immunoprecipitated the wild-type and phosphorylated forms of MDM2, we found that more p300 bound to the phosphorylated forms of MDM2. These results indicate that Akt can phosphorylate MDM2 at Ser166 and Ser186, and that the phosphorylated MDM2 has a stronger binding with p300 than the unphosphorylated form.

We also transfected wild-type MDM2 and phosphorylated forms of MDM2 (S166D, S186D or S166,186DD) into MDM2$^{-/-}$ and p53$^{-/-}$ cells with p14^{ARF}, and analysed the binding of these phosphorylated forms of MDM2 with p14^{ARF}. As shown in Fig. 7c, slightly less of the associated phosphorylated forms of MDM2 were present after immunoprecipitating the p14^{ARF} than of wild-type MDM2. The ARF protein binds directly to MDM2 to block p53 degradation by inhibiting the ubiquitin E3 ligase activity associated with MDM2 (ref. 27) and by sequestering MDM2 in nucleoli to prevent its export to the cytoplasm. There is persuasive evidence that ARF can inhibit the ubiquitin ligase activity of MDM2 *in vitro*, but exactly how ARF affects the nucleus–cytoplasm shuttling of MDM2 remains a matter of debate[15]. Current models propose that ARF function depends on its ability to sequester MDM2 in the nucleolus[15]. We examined whether the nucleoplasmic or nucleolus localization was different in wild-type and mutant MDM2 proteins. We transfected p53$^{-/-}$ and MDM2$^{-/-}$ MEF cells with p14^{ARF} together with wild-type or mutants of MDM2, and examined the nucleus and nucleolus localization of MDM2 by immunofluorescence analysis. As shown in Fig. 8, wild-type MDM2 was found

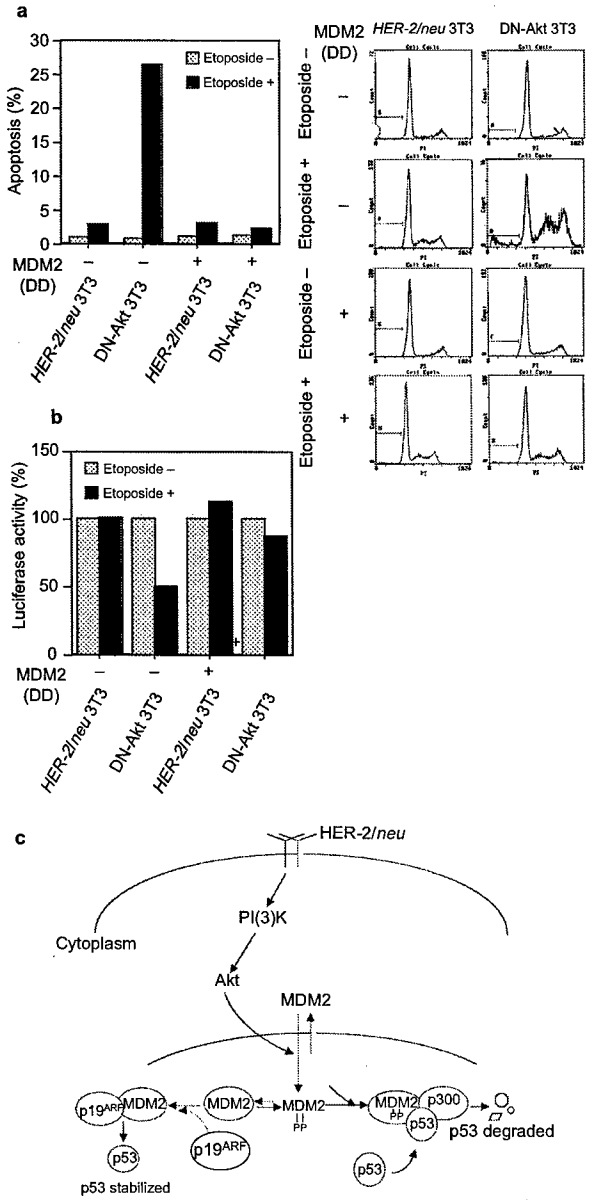

Figure 9 **Sensitization of etoposide-induced apoptosis by DN-Akt can be suppressed by nuclear MDM2 (S166,186DD). a**, Nuclear MDM2 blocks DN-Akt-mediated sensitization to etoposide-induced apoptosis. *HER-2/neu* 3T3 and DN-Akt 3T3 cells were transfected with a mimic phosphorylation form of MDM2 (S166,186DD) and then treated with etoposide (20 μM) for 48 h or left untreated. The apoptotic cells in each sample were analyaed by fluorescence-activated cell sorting as described in Fig. 1c. **b**, *HER-2/neu* 3T3 and DN-Akt 3T3 cells were transfected with MDM2 S166,186DD and a luciferase plasmid. After 12 h of transfection, cells were treated with 20 μM etoposide and incubated for another 36 h. The cell lysates were prepared and the luciferase activity in each sample was measured. **c**, A model showing how HER-2/*neu* induces the nuclear localization and altered binding of MDM2 with p300 and $p19^{ARF}$ via Akt.

uniformly in nucleoplasm in the presence or absence of $p14^{ARF}$. In the absence of MDM2, $p14^{ARF}$ was predominantly found in the nucleolus. Interestingly, MDM2 protein was not sequestered in nucleolus by $p14^{ARF}$, but rather brought the $p14^{ARF}$ out of nucleolus to the nucleoplasm. Our findings agree with recent studies that demonstrated that $p14^{ARF}$ can inhibit MDM2 function without relocalizing MDM2 to the nucleolus[35]. The cellular and subnuclear localization of MDM2 and ARF might depend on dynamic or static complex formation among MDM2, p53, ARF, Akt and some other proteins. Future systemic studies are required to clarify these issues. Taken together, our results indicate that Akt phosphorylates MDM2 at Ser166 and Ser186, and that the phosphorylated MDM2, which has a stronger binding with p300 and weaker association with $p19^{ARF}$, increases its nuclear localization and so leads to p53 degradation.

Sensitization of etoposide-induced apoptosis by DN-Akt can be suppressed by nuclear MDM2 (S166,186DD). As shown in Fig. 2, blocking the Akt pathway stabilizes p53 and therefore sensitizes cells to etoposide-induced apoptosis. MDM2 protein from the nuclear MDM2 mutant is found in the nucleus and is constitutively active without phosphorylation by Akt (Figs 5, 7). Thus, the nuclear MDM2 mutant should be able to suppress etoposide-induced apoptosis to which the cell is sensitized by blocking the Akt pathway. To address this issue, we transfected *HER-2/neu* 3T3 and DN-Akt 3T3 cells with the double mutant of MDM2 (S166,186DD) and measured their apoptosis after treatment with etoposide. As shown in Fig. 9a, the *HER-2/neu* 3T3 cells are resistant to etoposide-induced apoptosis, but blockage of the Akt pathway in DN-Akt 3T3 cells sensitizes the cells to apoptosis. However, when the S166,186DD mutant of MDM2 was transfected into DN-Akt 3T3 cells, the apoptosis induced by etoposide in DN-Akt 3T3 cells was dramatically suppressed. Similar results were obtained when we transfected with a luciferase reporter gene along with the double mutant of MDM2 and measured the luciferase activity after treatment with etoposide (Fig. 9b). Together, our results indicate that the sensitization of etoposide-induced apoptosis by the blockage of Akt pathway can be suppressed by a nuclear MDM2 mutant (S166,186DD).

Discussion

The tumour suppresser p53 plays a crucial role in controlling cell proliferation and apoptosis. In unstressed cells, p53 is latent and is maintained at low levels by targeted degradation mediated by its negative regulator, MDM2 (refs 14,17,20). The ability of MDM2 to ubiquitinate and degrade p53 depends on its E3 ligase activity and its ability to shuttle between the nucleus and the cytoplasm, which is regulated by its NLS and NES[20]. The transcriptional coactivator p300 has also been shown to form a ternary complex with MDM2 to facilitate the degradation of p53 (refs 22,23). However, the activity of MDM2 is negatively regulated by $p19^{ARF}$, which inhibits its E3 ligase activity[15]. Because the regions that control MDM2 cellular localization and binding with p300 and $p19^{ARF}$ overlap, the issues of when, where and how MDM2 is localized and interacts with functionally opposite molecules such as p300 and $p19^{ARF}$ are crucial to understanding the function of MDM2 and its regulation of p53.

In this study, we have identified a novel mechanism that controls the cellular localization of MDM2 and its interaction with p300 and $p19^{ARF}$. We show that Akt physically associates with MDM2 and phosphorylates it at Ser166 and Ser186. Our results, together with previous findings[14–29], lead us to propose a model for how Akt affects the ubiquitination and degradation of p53 (Fig. 9c). Akt phosphorylates MDM2 and induces its nuclear translocation, and the phosphorylated MDM2 binds p300 but not to $p19^{ARF}$. Binding to p300 provides a platform to facilitate the assembly of the phosphorylated MDM2–p300–p53 complex and leads to the ubiquitination and degradation of p53. Blocking the Akt pathway with DN-Akt prevents the nuclear translocation of MDM2. The unphosphorylated MDM2 in the nucleus only binds $p19^{ARF}$ but not to p300. Binding to $p19^{ARF}$ inhibits MDM2's ubiquitin E3 ligase activity and consequently stabilizes the p53 protein.

Table 1 Immunoreactivity of MDM2, Akt-p and HER-2/*neu* in surgical specimens of breast cancer

	HER-2/*neu* and Akt-p staining		
MDM2 staining	Negative (n = 12)	Positive (n = 21)	Total
Mainly nucleus	1 (3%)	16 (49%)	17 (51%)
Mainly cytoplasm	11 (33%)	5 (15%)	16 (49%)
Total	12 (36%)	21 (64%)	33 (100%)

The 33 surgical specimens of breast cancer were stained for *HER-2/neu*, phosphorylation form of Akt and MDM2 as shownin Fig. 6. The expression patterns of these three molecules in the samples from each patient was determined and summarized. Correlation of subcellular localization of MDM2 and the expression of phosphorylation of Akt was analysed by using Fisher's exact test ($P < 0.001$). A P value of less than 0.05 was set as the criterion for statistical significance.

It is evident that many of the tumours with wild-type p53 show defects in the ability to induce a p53 response. Overexpression of MDM2 is found in many types of tumour[16]. In addition, $p19^{ARF}$, the negative regulator of MDM2, is also often deleted or mutated in a wide spectrum of cancers[15]. Thus, overexpression of *MDM2* and the lack of $p19^{ARF}$ in cancers greatly increase the ability of MDM2 to degrade p53, and cause a problem for gene therapy using p53 constructs. Overexpression of *HER-2/neu* is known to activate the Akt pathway and to confer resistance to the apoptosis induced by many chemotherupeutic drugs[30]. We show that blocking the Akt pathway inhibits the nuclear translocation of MDM2 and the binding of p300, and allows the binding of $p19^{ARF}$, so sensitizing the *HER-2/neu*-overexpressing cells to DNA-damaging agents. Our results might open an avenue for developing novel anticancer therapies for *HER-2/neu*-overexpressing or Akt-activating cancers.

Note added in proof: Mayo and Donner[36] have recently reported that the PI(3)K–Akt pathway promotes the translocation of MDM2 from the cytoplasm to the nucleus. □

Methods

Materials.

The DNA-damaging agents etoposide and doxorubicin were purchased from Calbiochem. The DNA dye DAPI and anti-HA (12C5) antibody were purchased from Roche Molecular Biochemicals. The anti-p53 antibodies Ab-1 and Ab-7, and the anti-MDM2 antibodies SMP 14 and 2A10, were obtained from Oncogene Research; the anti-p300 and anti-$p19^{ARF}$ antibodies were from Santa Cruz Biotechnology. Female nude mice (*Nu/Nu*, 6 weeks old) were purchased from Harlan (Indianapolis, Indiana).

MDM2 constructs.

A *Bam*HI and an *Eco*RI site were generated near the start and termination codons, respectively, in human *MDM2* by PCR and subcloned into the expression vector pcDNA3. Site-directed mutagenesis was performed as described previously[5]. Ser166 and Ser186 in MDM2 were replaced by Asp by using the following primers: for S166D, 5′-CTAGAAGGAGAGCAATTGATGAGACAGAAGAAAATTC-3′; and for S186D, 5′-GAAAACGCCACAAAGATGATAGTATTTCCC-3′. The sequences of the wild-type and mutant *MDM2* constructs were verified by automated sequencing. To generate wild-type and mutant *MDM2* GST-tagged bacterial expression constructs, the same fragments containing the wild-type and mutant *MDM2* were subcloned into the bacterial expression vector pGEX4T-3 (Pharmacia). Expression of wild-type and mutant MDM2 proteins was induced in *Escherichia coli* strain BL21 and purified by glutathione–Sepharose chromatography (Pharmacia).

Cell culture.

NIH3T3, *HER-2/neu* 3T3, p53 and MDM2-deficient MEF, and 293T cells were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% foetal bovine serum. The DN-Akt transfectants of *HER-2/neu* 3T3 cells were grown under the same conditions except that 200 μg ml^{-1} G418 was added to the culture medium[4]. The 293T cells were transfected by the calcium phosphate technique and p53- and MDM2-deficient cells by the liposome method.

In vitro growth rate analysis.

The *in vitro* growth rates of the cell lines were assessed by the MTT assay as described previously[5].

[^{3}H]-Thymidine incorporation assay.

The proliferation rates of the cell lines were analysed by measuring [^{3}H]-thymidine incorporation as described previously[5].

In vivo tumour growth analysis.

The nude mice were randomly divided into four groups of five mice each. Two groups of mice were subcutaneously inoculated with 5×10^5 cells of *HER-2/neu* 3T3 or DN-Akt 3T3 cells per mouse. On days 5 and 7 after inoculation, one group from each cell line was giving two intravenous injections of etoposide (20 mg kg^{-1}) via the tail vein. Another two groups of mice were injected with the same volume of normal saline (the solvent for etoposide) as a control. The tumour volume (V) was measured every 2 days and calculated as $V = 0.5 \times ab^2$, where a and b are the longest and shortest diameters of the tumour, respectively. The experiment was stopped 15 days after inoculation and the results were tested statistically using two-sided log-rank analysis.

In vitro kinase assay.

293T cells (2×10^5) were transfected with 20 μg of HA-tagged CA-Akt or DN-Akt. 48 h after transfection, Akt was immunoprecipitated from cell extracts and incubated with 5 μg of purified GST–MDM2 (wild-type or mutant) in the presence of 5 μCi of [γ-^{32}P]ATP and 50 mM cold ATP in a kinase buffer for 30 min at 30 °C as described previously[5]. The reaction products were resolved by SDS-PAGE and the ^{32}P-labelled proteins were visualized by autoradiography.

Immunoprecipitation and immunoblotting.

Cells were washed twice with PBS and scraped into 500 μl of RIPA buffer. After a brief sonication, the lysate was centrifuged at 14,000 *g* for 5 min at 4 °C to remove the insoluble cell debris. Immunoprecipitation and immunoblotting were performed as described previously[4].

In situ immunofluorescent staining.

~2×10^4 cells of *HER-2/neu* 3T3 or DN-Akt 3T3 cells were plated into chamber slides for 16 h. After fixation in 4% paraformaldehyde for 1 h and permeabilization with 0.2% Triton X-100 for 30 min, the cellular localization of MDM2 was determined by using a monoclonal antibody against MDM2 (SMP14) diluted 1:1000. After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG (diluted 1:500) plus Dapi (0.1 μg ml^{-1}) for 1 h. After extensive washing, the samples were examined under a fluorescent microscope (Zeiss). Nonspecific reaction of the secondary antibody was ruled out by the absence of fluorescence under the microscope.

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Akt takes centre stage in cell-cycle deregulation

Wafik S. El-Deiry

The universal cell-cycle inhibitor $p21^{Cip1/WAF1}$ is phosphorylated and localized in the cytoplasm in Her2/neu-overexpressing breast cancers as a result of its physical association with the oncogenic Akt protein. Subcellular mislocalization of checkpoint controllers is now surfacing as a mechanism of deregulating cell proliferation in cancer.

Abnormal gene amplification and increased cell-surface expression of the transmembrane Her2/neu oncoprotein receptor (relative molecular mass 185,000; also known as ErbB2) occurs in up to 30% of human breast and other cancers (reviewed in ref. 1). Neu was originally discovered as an oncogene product in chemically-induced neuroblastomas in rats. The Her2/neu receptor, a member of the 'growth factor' glycoprotein family that includes the epidermal growth-factor receptor (EGFR), possesses a cytoplasmic tyrosine-kinase domain that signals cell proliferation and increased cell survival. Moreover, Her2/neu overexpression has been associated with poor patient prognosis and poor response to chemotherapeutic agents such as Taxol. Recent excitement has surrounded the clinical development and remarkable therapeutic effects of Her2/neu-blocking antibodies, such as Herceptin[2]. Meanwhile, the molecular details of how Her2/neu signals enhance growth and survival of cancer cells and induce therapeutic resistance continue to emerge[3]. On page 245 of this issue, Zhou *et al.*[4], describe an elegant mechanism by which Her2/neu and its target oncoprotein Akt subvert the principal negative cell-cycle controller $p21^{Cip1/WAF1}$ to enhance proliferation and survival of cancer cells (Fig. 1).

The cell-survival-promoting oncoprotein Akt (also known as protein kinase B, PKB) is abnormally activated in several human malignancies and can suppress apoptosis[5,6], although data obtained recently from a mouse knockout model indicate, surprisingly, that the p110γ catalytic subunit of phosphoinositide-3-OH kinase (PI(3)K; an upstream activator of Akt) may suppress colon tumorigenesis[7]. Overexpression of the *Drosophila* Akt homologue regulates cell and organ size without significantly affecting cell number or proliferation[8]. The *AKT1* gene encodes a serine/threonine kinase, the activity of which is induced upon recruitment to the plasma membrane and phosphorylation by regulatory kinases[9]. Recruitment and activation of Akt requires the presence of the phosphoinositide triphosphate (PI-3,4,5-P). Increases in PI-3,4,5-P levels (in response to PI(3)K activation) and Akt kinase activity occur in response to a variety of growth and survival signals including insulin, platelet-derived growth factor (PDGF), EGFR, Ras and Her2/neu, and the Akt signalling pathway is negatively regulated by the PTEN tumour-suppressor lipid phosphatase[8,10,11]. Downstream targets of Akt kinase that mediate its proliferative and pro-survival effects include Bad, caspase-9, Forkhead and IκB kinase-α in survival signalling, and p27 and GSK3β/cyclin D1 in pathways that control the cell cycle[9,11].

Remarkable progress has been made in determining how the molecular engine that drives the cell cycle is controlled and in identifying its aberrant function in human cancer. In eukaryotes, carefully orchestrated cell-cycle transitions depend on the ordered assembly, activation and activity of several conserved cyclin-dependent kinase (CDK) complexes, which phosphorylate key substrates such as the retinoblastoma protein family in G1 phase, the origin-recognition complex (ORC) and E2F1 in S phase, and Cdc25C in the G2/M-phase transition. CDK function is regulated at the levels of cyclin synthesis, formation of cyclin–CDK complexes, phosphorylation of activating and inhibitory residues on the CDK kinase, and subcellular localization of cyclin–CDK complexes, and by two families of cell-cycle inhibitory proteins (known as CDK inhibitors or CDKIs; Table 1)[12]. Cyclin–CDK complexes are the ultimate targets of several cell-cycle checkpoints that ensure the maintenance of genomic integrity and cellular good health[13,14]. One hallmark of cancer cells is the failure of such checkpoints, for example in response to improper mitotic-spindle assembly, DNA damage, starvation or oncogene activation; this allows DNA-replication errors and chromosomal abnormalities to be propagated and to accumulate over time because of defective monitoring and repair of the genetic material during cell division. CDKIs were first identified in the early 1990s through a convergence of work in several fields, including cancer biology, cell cycle and the genetics of cellular senescence. $p21^{Cip1/WAF1}$, the first CDKI to be identified, is a transcriptional target of the p53 tumour suppressor during the stress response and seems to be regulated by p53-independent transcription factors during growth arrest associated with cellular differentiation. There is some evidence that loss or haploinsufficiency of $p21^{Cip1/WAF1}$ contributes to checkpoint defects as a result of DNA damage or nucleotide depletion, cellular transformation, tumorigenesis or altered potential for stem-cell repopulation[15–19].

A connection between Her2/neu overexpression, increased $p21^{Cip1/WAF1}$ expression, and resistance of breast cancers to the microtubule-arresting agent Taxol was previously noted by Yu *et al.*[20]. Increased resistance to Taxol was reversed by antisense inhibition of $p21^{Cip1/WAF1}$ expression in cells overexpressing Her2/neu, and Her2/neu failed to sensitize p21-null mouse embryonic fibroblasts to the killing effects of Taxol. The authors proposed that the modest reduction

Table 1 Consequences of CDKI inactivation

CDK inhibitor (CDKI)	Defect in human cancer	Consequences for CDKI
$p16^{INK4A}$	Mutation	Loss of function
	Hypermethylation	Loss of expression
	Deletion	Loss of expression
$p27^{KIP1}$	Mutation	Loss of function
	TGF-β receptor mutation	Reduced function
	Hyperubiquitination	Loss of expression
$p21^{Cip1/WAF1}$	p53 mutation	Reduced expression
	c-Myc overexpression	Transcriptional repression
	Her2/neu overexpression	Cytoplasmic localization
$p57^{KIP2}$	Imprinting	Reduced expression

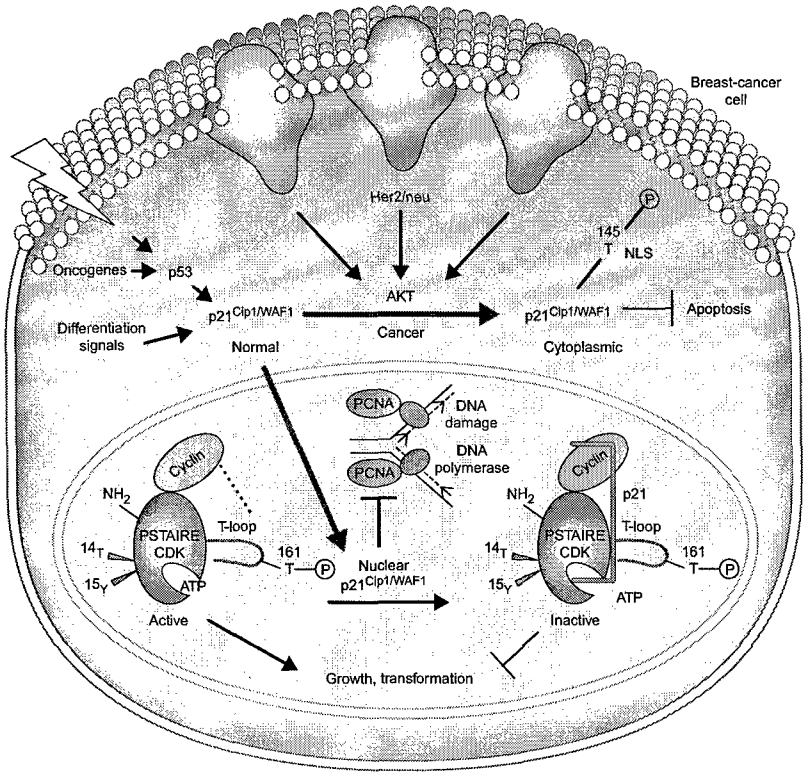

Figure 1 **Oncogene Her2/neu-initiated, Akt-mediated p21$^{Cip1/WAF1}$ inactivation as a suppressor of tumour growth and the cell cycle. During normal cellular growth, the p21$^{Cip1/WAF1}$ CDK inhibitor is expressed at low levels and aids assembly of growth-promoting active cyclin–CDK complexes. Under cellular stress, p21$^{Cip1/WAF1}$ expression is increased through p53-dependent and -independent pathways. Increased p21$^{Cip1/WAF1}$ levels in the nucleus lead to inhibition of CDKs through binding of p21$^{Cip1/WAF1}$ to the complexes. Binding of p21Cip1/WAF1 to cyclin–CDK leads to a conformational change in CDKs, and to steric hindrance of ATP binding to the CDK active site. The C terminus of p21$^{Cip1/WAF1}$, containing its nuclear localization signal (NLS), binds to and inhibits proliferating-cell nuclear antigen (PCNA), thereby blocking DNA replication. In many cancers, the checkpoint function of p21$^{Cip1/WAF1}$ is lost through mutations in its chief regulator during cellular stress, p53. In cancers that overexpress Her2/neu, Akt is also activated. Zhou and colleagues have now identified p21$^{Cip1/WAF1}$ as an Akt-interacting protein and a substrate for phosphorylation in Her2/neu-overexpressing cancers.**

in Cdc2 kinase activity they observed in cells with increased p21$^{Cip1/WAF1}$ levels led in part to the survival of Taxol-treated Her2/neu-overexpressing cells. However, it remained unexplained how p21$^{Cip1/WAF1}$ expression could be greatly increased at the same time as an increase in cell proliferation associated with oncogene Her2/neu overexpression, in the absence of Taxol.

An important clue in the signalling pathway downstream of Her2/neu was uncovered when Zhou and colleagues began investigating the contribution of Akt signalling to Her2/neu-induced cell proliferation. They found that dominant negative Akt or inhibitors of PI(3)-kinases inhibited the proliferation of Her2/neu-overexpressing cells but had no effect on the greatly increased p21$^{Cip1/WAF1}$ expression. These findings indicate that Her2/neu, through Akt, must somehow inactivate the growth-inhibiting effect of p21$^{Cip1/WAF1}$ without altering its expression. As Akt is known to affect the subcellular localization of some proteins, such as the Forkhead transcription factor, through phosphorylation, and as p21$^{Cip1/WAF1}$ contains a potential Akt consensus site, the authors investigated whether p21$^{Cip1/WAF1}$ might be a direct target for Akt phosphorylation. They found that endogenous Akt physically associates with endogenous p21$^{Cip1/WAF1}$ and phosphorylates it at threonine 145, causing p21$^{Cip1/WAF1}$ to be localized in the cytoplasm.

To understand better the role of p21$^{Cip1/WAF1}$ phosphorylation in growth suppression, Zhou and colleagues introduced alanine and aspartate residues at position 145 of human p21. The T145A mutant of p21$^{Cip1/WAF1}$, unlike the wild type, effectively suppressed growth and DNA synthesis in NIH 3T3 cells when Her2/neu was overexpressed, presumably because the mutant was no longer regulated by the kinase activity of Akt. In contrast, the T145D mutant was unable to suppress growth efficiently or to inhibit DNA synthesis under any circumstances, especially in the presence of dominant negative Akt — a condition under which wild-type p21$^{Cip1/WAF1}$ was effective. As the authors predicted, the T145A and T145D mutants of p21$^{Cip1/WAF1}$ were localized predominantly in the nucleus and the cytoplasm, respectively. As an important test of whether the observed phosphorylation of p21$^{Cip1/WAF1}$ might be relevant to tumours derived from breast-cancer patients, the authors obtained convincing evidence by correlating Her2/neu overexpression with Akt phosphorylation and cytoplasmic p21$^{Cip1/WAF1}$ localization in ten different tumours. These *in vivo* results add further support to the idea that activation of Akt kinase downstream of Her2/neu overexpression may be responsible for mislocalization of p21$^{Cip1/WAF1}$ to the cytoplasm and disruption of its potent growth-inhibiting activity.

There are potentially interesting parallels between breast and prostate cancer in terms of the role of Her2/neu and Akt signalling in survival and hormone-independent growth. In breast cancer, there is a well known reciprocal relationship between Her2/neu overexpression, loss of oestrogen-receptor expression and poor prognosis[20]. In prostate cancer, recent work has shown that Akt, acting downstream of overexpressed Her2/neu, binds to and phosphorylates the androgen receptor, leading to androgen-independent growth and survival[21]. It is possible that, in prostate cancer, activated AKT may also deregulate cell-cycle control and enhance survival after cytotoxic exposure through effects on p21$^{Cip1/WAF1}$.

The identification of p21$^{Cip1/WAF1}$ as a target for Akt provides new insights and new avenues for understanding cell-cycle regulation, and indicates potential strategies for therapeutic intervention in cancer. It would be of interest to determine the physiological or pathological circumstances in which p21$^{Cip1/WAF1}$ is phosphorylated and what impact this has on its function. In this regard, animal models in which p21$^{Cip1/WAF1}$ has been found to contribute to differentiation or tumour suppression may prove useful for investigating the effects of knock-in of p21$^{Cip1/WAF1}$ phosphorylation-site mutants. Studying how phosphorylated p21$^{Cip1/WAF1}$ aids cyclin–CDK assembly may

provide insights into potential functions of $p21^{Cip1/WAF1}$ within the cytoplasm. The interactions of phosphorylated $p21^{Cip1/WAF1}$ may modulate cytoplasmic kinase activities that influence the stress response or cell-death pathways. In this regard, it would be of interest to test $p21^{Cip1/WAF1}$ mutants that are localized to the cytoplasm but that are otherwise impaired in cytoplasmic interactions; that is, is the loss of nuclear localization sufficient for the Her2/neu-overexpression and drug-resistance phenotype, or is there also a gain-of-function in the cytoplasm? It would also be of interest to determine the extent to which $p21^{Cip1/WAF1}$ phosphorylation is required for Akt effects on the cell-cycle, compared with inhibition of cell death, given earlier reports of the effects of Akt on $p27^{KIP1}$ and cyclin D1 in cell-cycle deregulation.

In terms of therapeutic intervention, small peptides may be designed on the basis of the carboxy-terminal structure of $p21^{Cip1/WAF1}$, which may compete with endogenous $p21^{Cip1/WAF1}$ for Akt binding and phosphorylation, thus sending $p21^{Cip1/WAF1}$ back into the nucleus. Such strategies might take advantage of the increased levels of $p21^{Cip1/WAF1}$ in Her2/neu-overexpressing cells to suppress growth. Because increased $p21^{Cip1/WAF1}$ expression might be expected to be lost after treatment with Herceptin, it may be useful to compare the cell-cycle-inhibiting and drug-sensitization effects of blocking the Her2/neu pathway proximally, with effects achieved by blocking specific downstream effectors of Akt signalling. It may also be fruitful to investigate how Her2/neu increases $p21^{Cip1/WAF1}$ expression, with the aim of developing strategies to control the cell cycle while blocking other oncogenic Her2/neu signals. It may be possible to bypass the effect of Akt on $p21^{Cip1/WAF1}$ through the use of small-molecule CDK inhibitors. Knowledge of the Her2/neu-AKT-$p21^{Cip1/WAF1}$ pathway may be of use in designing more effective cytotoxic therapeutic strategies for Her2/neu-overexpressing or PTEN-mutated cancers. For example, agents that induce DNA damage and p53-dependent $p21^{Cip1/WAF1}$ upregulation might be used more effectively to treat wild-type p53-expressing, Her2/neu-overexpressing cancers if $p21^{Cip1/WAF1}$ were rerouted back to the nucleus. Another potential strategy might involve screening for agents that upregulate phosphatases that dephosphorylate $p21^{Cip1/WAF1}$, thus reactivating its nuclear-localization signal. It is clear that Akt is a focal point for deregulation in cancer and can have impact on cell proliferation and survival of cancer cells through a growing list of key targets that now includes $p21^{Cip1/WAF1}$.

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Cytoplasmic localization of p21$^{Cip1/WAF1}$ by Akt-induced phosphorylation in *HER-2/neu*-overexpressing cells

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Amplification or overexpression of *HER-2/neu* in cancer cells confers resistance to apoptosis and promotes cell growth. The cellular localization of p21$^{Cip1/WAF1}$ has been proposed to be critical either in promoting cell survival or in inhibiting cell growth. Here we show that *HER-2/neu*-mediated cell growth requires the activation of Akt, which associates with p21$^{Cip1/WAF1}$ and phosphorylates it at threonine 145, resulting in cytoplasmic localization of p21$^{Cip1/WAF1}$. Furthermore, blocking the Akt pathway with a dominant-negative Akt mutant restores the nuclear localization and cell-growth-inhibiting activity of p21$^{Cip1/WAF1}$. Our results indicate that *HER-2/neu* induces cytoplasmic localization of p21$^{Cip1/WAF1}$ through activation of Akt to promote cell growth, which may have implications for the oncogenic activity of *HER-2/neu* and Akt.

The *HER-2/neu* gene (also known as *c-erbB2*) encodes a transmembrane receptor tyrosine kinase of relative molecular mass 185,000 (M_r 185K) that shares partial homology with the other members of the family of epidermal growth-factor receptors. Amplification or overexpression of *HER-2/neu* occurs in ~30% of human breast and ovarian cancers and is a marker of poor prognosis[1–3]. We have previously shown that *HER-2/neu* activates the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway and confers resistance to apoptosis induced by tumour-necrosis factor[4]. The PI-3K/Akt pathway has an important role in preventing cells from undergoing apoptosis and contributes to the pathogenesis of malignancy[5,6]. For example, activated Akt phosphorylates specific targets such as Bad[7], caspase-9 (ref. 8), forkhead transcription factors[9,10] and IKK-α (refs 11, 12), thereby promoting cell survival. However, in addition to its anti-apoptotic function, Akt is also involved in cell proliferation[13–15]. Furthermore, Akt detaches from the inner surface of the plasma membrane, where it is initially activated, and relocalizes to the nucleus within 30 min of activation by growth factors[16,17]. These findings indicate that some critical Akt targets that control cell-cycle progression may be located within the nucleus.

Cell-cycle progression is tightly regulated by the family of cyclin-dependent kinase (CDK) inhibitors. p21$^{Cip1/WAF1}$ was identified through its interaction with Cdk2 (ref. 18), and its expression is induced by activation of wild-type p53 (ref. 19), and during cellular senescence[20]. The cell-growth-inhibiting activity of p21$^{Cip1/WAF1}$ is strongly correlated with its nuclear localization[21,22]. However, recent evidence has shown that p21$^{Cip1/WAF1}$ can also localize in the cytoplasm and has an important role in protecting cells against apoptosis. For instance, nuclear p21$^{Cip1/WAF1}$ translocates to the cytoplasm after differentiation of U937 cells into monocytes, and this translocation event is accompanied by resistance to various apoptotic stimuli[23]. Furthermore, cytoplasmic p21$^{Cip1/WAF1}$ forms a complex with apoptosis-signal-regulating kinase 1 (ASK1) that inhibits the stress-induced mitogen-activated protein (MAP) kinase cascade and therefore results in resistance to apoptosis in these cells[23]. However, the mechanism that regulates the localization of p21$^{Cip1/WAF1}$ is still unknown.

Here we show that blocking the Akt pathway by using a dominant negative Akt mutant (DN-Akt) inhibits cell growth. This growth inhibition is correlated with nuclear localization of p21$^{Cip1/WAF1}$. We demonstrate that Akt can associate with p21$^{Cip1/WAF1}$ and phosphorylates a consensus threonine residue (T145) in the nuclear-localization signal (NLS) of p21$^{Cip1/WAF1}$, leading to the cytoplasmic localization of p21$^{Cip1/WAF1}$. We thus identify a new signalling pathway and show that overexpression of *HER-2/neu* may enhance cell proliferation by inducing cytoplasmic localization of p21$^{Cip1/WAF1}$ through the serine/threonine kinase Akt.

Results

The Akt pathway is required for *HER-2/neu*-mediated cell proliferation. To study the effect of Akt on *HER-2/neu*-mediated cell proliferation, we used a model system that consists of NIH3T3 cells, *HER-2/neu* 3T3 cells (NIH3T3 cells transformed with *HER-2/neu*) and DN-Akt 3T3 cells (*HER-2/neu* 3T3 cells transfected with DN-Akt)[4]. As expected, *HER-2/neu* 3T3 cells grew much faster than the parental NIH3T3 cells (Fig. 1a). The Akt pathway is known to be constitutively activated in *HER-2/neu* 3T3 cells[4], and when this pathway was blocked by DN-Akt, cell growth became slower (Fig. 1a). This was not due to the heterogeneity of the cell clones, because a specific PI-3K inhibitor, wortmannin, also produced a similar slowing of growth in *HER-2/neu* 3T3 cells (Fig. 1a). When the DNA synthesis rate was determined by measuring incorporation of [^{3}H]thymidine, *HER-2/neu* 3T3 cells also exhibited greater amounts of DNA synthesis than the parental NIH3T3 cells (Fig. 1b). *HER-2/neu*-induced DNA synthesis was significantly inhibited by blocking the Akt pathway with either wortmannin or DN-Akt.

As the net rate of cell growth depends on a fine balance between the rates of cell proliferation and cell death, we also investigated whether apoptosis contributes to the difference in growth in these cells. There was no significant difference in apoptosis among these cells, as measured by fluorescence-activated cell sorting (FACS) analysis (Fig. 1c). Therefore, the reduction in cell growth in DN-Akt 3T3 cells was most probably a result of the reduction in cell proliferation. To investigate further, we also carried out the same experiments using another *HER-2/neu*-overexpressing breast-cancer cell line, MDA-MB453, and stable DN-Akt transfectants of it[4]. As in *HER-2/neu* 3T3 and DN-Akt 3T3 cells, three independent clones of DN-Akt transfectants showed reductions in cell growth and DNA synthesis (Fig. 1d, e), but no difference was observed in apoptosis (FACS analysis, data not shown). A revertant that had lost DN-Akt during culture exhibited rates of cell growth and DNA synthesis that were almost identical to those of parental MDA-

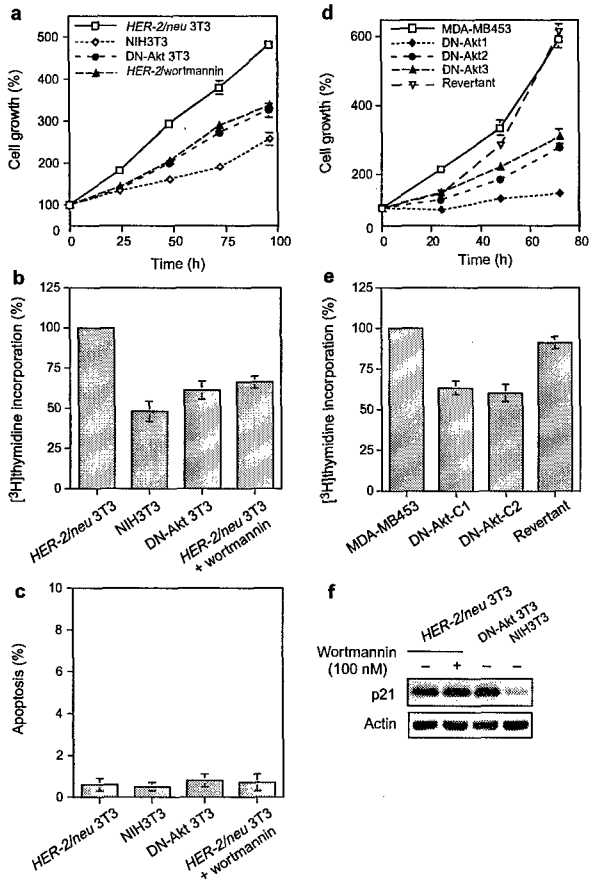

Figure 1 **The Akt pathway is required for *HER-2/neu*-mediated cell proliferation. a**, Blocking the Akt pathway reduces the growth of *HER-2/neu* 3T3 cells. Cells (3×10^3) were seeded in 96-well plates and grown in DMEM/F12 plus 1% FBS. Growth rates were monitored by the MTT assay. Data are means ± s.e.m. from three independent experiments, each carried out in quadruplicate. **b**, Cells (3×10^3) were grown as in **a** with 1 μCi [^{3}H]thymidine for 12 h. Rates of cell replication were determined by measuring [^{3}H]thymidine incorporation. Data are means ± s.e.m. from three independent experiments, each carried out in quadruplicate. **c**, Blocking the Akt pathway does not significantly induce apoptosis in *HER-2/neu* 3T3 cells. Cells were grown in 1% serum, and the percentage of cells in apoptosis was measured by FACS as described[4]. Data are means ± s.e.m. from three independent experiments. **d**, Blocking the Akt pathway reduces cell growth in MDA-MB453 cells. Cells were grown in six-well plates as in **a**, and their growth rates were measured by cell counting (see Methods). Data are means ± s.e.m. from three independent experiments. **e**, Rates of DNA synthesis in each of the cell lines in **d** was determined as in **b**. **f**, Overexpression of *HER-2/neu* induces expression of p21$^{Cip1/WAF1}$. Lysates (50 μg) from the indicated cell lines were analysed by 12% SDS–PAGE. After proteins were transferred to a nitrocellulose membrane, expression of p21$^{Cip1/WAF1}$ was measured using a monoclonal antibody against p21$^{Cip1/WAF1}$ (Pharmingen).

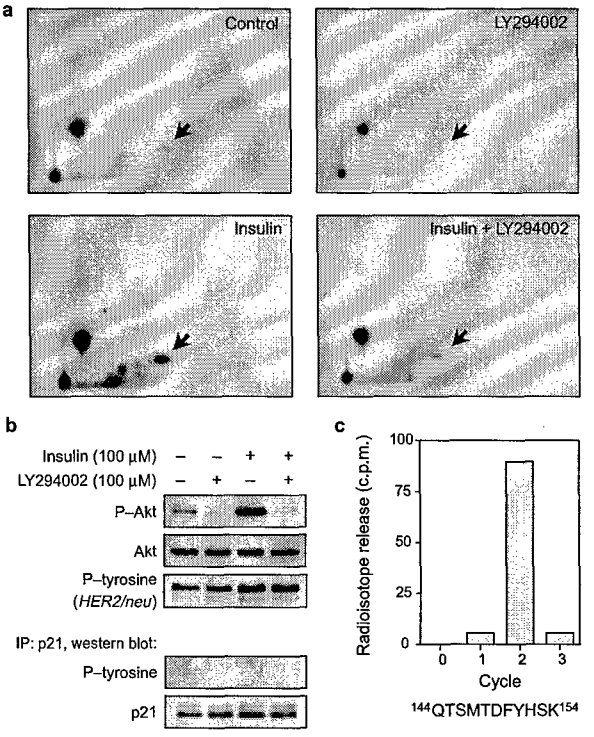

Figure 2 **Threonine 145 of p21$^{Cip1/WAF1}$ is phosphorylated *in vivo*. a**, Endogenous p21$^{Cip1/WAF1}$ is phosphorylated *in vivo* in response to insulin. MDA-MB453 cells were incubated with 1.5 mCi ml^{-1} [^{32}P]orthophosphate for 3.5 h and then stimulated for 30 min with insulin in the presence or absence of LY294002. Cells were then lysed, and the endogenous p21$^{Cip1/WAF1}$ was immunoprecipitated with a specific antibody and subjected to two-dimensional phosphopeptide analysis (see Methods). Arrows indicate the spots induced by insulin and inhibited by PI-3K inhibitor. **b**, Tyrosine residues in p21$^{Cip1/WAF1}$ are not phosphorylated. MDA-MB453 cells were treated as in **a**, and one-third of the lysate was analysed by western blotting for activation of Akt and tyrosine phosphorylation of *HER-2/neu* (exposure to anti-phosphotyrosine antibody clone 4G10 (Upstate Biotechnology) for 2 s). The remaining lysate was subjected to immunoprecipitation (IP) with a specific antibody against p21$^{Cip1/WAF1}$, and to western blotting with specific antibodies against p21$^{Cip1/WAF1}$ and phosphotyrosine (exposure to clone 4G10 for 20 min). P denotes phosphorylation. **c**, p21$^{Cip1/WAF1}$ is phosphorylated at T145. MDA-MB453 cells were treated as in **a**, and p21$^{Cip1/WAF1}$ was immunoprecipitated and subjected to trypsin digestion and to amino-acid sequencing by Edman degradation (the sequence of the relevant peptide is shown at the bottom). The amount of radioisotope released from each cycle of Edman degradation was detected with a Cerenkov counter. Background counts, which were typically 50–60 c.p.m., were subtracted from each measurement.

MB453 cells (Fig. 1d, e). Together, our results indicate that the Akt pathway is required for *HER-2/neu*-mediated cell proliferation and that inhibition of this pathway, either by a PI-3K inhibitor or by DN-Akt, significantly reduces cell proliferation.

Cell proliferation is tightly regulated by the family of CDK inhibitors. For example, p21$^{Cip1/WAF1}$ has been shown to have a critical function in regulating cell proliferation[18–20]. We therefore monitored expression of p21$^{Cip1/WAF1}$ in the cell lines described above. Levels of p21$^{Cip1/WAF1}$ expression were significantly higher in *HER-2/neu* 3T3 cells than in the parental cells (Fig. 1f), which is consistent with previous results[24]. Inhibition of the Akt pathway by DN-Akt did not significantly affect p21$^{Cip1/WAF1}$ expression (Fig. 1f). This result cannot account for the *HER-2/neu*-induced cell proliferation and indicates that there may be an unknown mechanism that overrides that of enhanced expression of p21$^{Cip1/WAF1}$ in *HER-2/neu* 3T3 cells.

Threonine 145 of p21$^{Cip1/WAF1}$ is phosphorylated by Akt. As protein phosphorylation has a significant role in the regulation of protein function, and as p21$^{Cip1/WAF1}$ has been shown to be phosphorylated *in vitro* by protein kinase C (ref. 25), we examined the phosphorylation pattern of endogenous p21$^{Cip1/WAF1}$, using *in vivo* [^{32}P]orthophosphate labelling and two-dimensional phosphopeptide analysis (Fig. 2a). A strong phosphorylated peptide spot was observed after induction by

insulin, which is known to activate PI-3K/Akt; this spot was much weaker in cells that were incubated with the PI-3K inhibitor LY294002. This insulin-induced phosphorylation spot was most probably a result of phosphorylation of serine or threonine, rather than tyrosine, residues in $p21^{Cip1/WAF1}$, because no tyrosine phosphorylation was detected when endogenous $p21^{Cip1/WAF1}$ was immunoprecipitated and examined by western blotting with a specific antibody against phosphorylated tyrosine (Fig. 2b). The fact that no tyrosine-phosphorylated endogenous $p21^{Cip1/WAF1}$ was detected was not due to the quality of the antibody, because the same antibody was able to detect tyrosine phosphorylation of the *HER-2/neu* gene product in the same cell lysate (Fig. 2b). We investigated further the site of insulin-induced phosphorylation in $p21^{Cip1/WAF1}$ by *in vivo* [^{32}P]orthophosphate labelling, and then subjected the trypsin-digested phosphopeptides to amino-acid sequencing by Edman degradation. The activity of radioisotope was released after the second cycle of Edman degradation (Fig. 2c), but was greatly reduced when cells were pretreated with LY294002 (data not shown). Judging from the amino-acid sequence of $p21^{Cip1/WAF1}$, the only trypsin-digested peptide that contains threonine or serine at position 2 in its sequence is ^{144}QTSMTDFYHSK^{154}. Thus, our results indicate that T145 of $p21^{Cip1/WAF1}$ is phosphorylated *in vivo* and that this phosphorylation could be induced by insulin and inhibited by PI-3K inhibitor.

The nuclear localization of $p21^{Cip1/WAF1}$ seems to be responsible for its inhibition of cell growth and is controlled by the NLS at its carboxy terminus[26]. We noticed that there is a putative Akt-phosphorylation motif, containing the phosphorylated T145 residue (Fig. 2) in the NLS of $p21^{Cip1/WAF1}$ and that motif is highly conserved among different species (Fig. 3a). To determine whether Akt interacts with and phosphorylates $p21^{Cip1/WAF1}$ and so regulates its cellular localization, we first carried out co-immunoprecipitation experiments to detect an association between Akt and $p21^{Cip1/WAF1}$. After immunoprecipitating endogenous Akt from MDA-MB453 cells, we detected the presence of endogenous $p21^{Cip1/WAF1}$, whereas no endogenous $p21^{Cip1/WAF1}$ was detected using a non-specfic antibody (Fig. 3b). We also co-transfected 293T cells with constitutively active Akt (CA-Akt) or DN-Akt together with wild-type or mutant $p21^{Cip1/WAF1}$ (the mutant containing a T145A mutation). After immunoprecipitating $p21^{Cip1/WAF1}$, we detected Akt and vice versa (Fig. 3c, d), indicating that these two molecules are associated. This association was dependent on the phosphorylation status of Akt and on the status of $p21^{Cip1/WAF1}$. $p21^{Cip1/WAF1}$ associated more strongly with CA-Akt than with DN-Akt, and un-phosphorylated $p21^{Cip1/WAF1}$(T145A) associated much more weakly with Akt than did wild-type $p21^{Cip1/WAF1}$. Furthermore, Akt could phosphorylate $p21^{Cip1/WAF1}$ both *in vitro* and *in vivo*, whereas DN-Akt could not (Fig. 3e, f). The fact that no phosphorylation was observed on $p21^{Cip1/WAF1}$(T145A) indicates that Akt interacts with $p21^{Cip1/WAF1}$ and phosphorylates it at T145. This is further supported by the fact that phosphorylation at T145 is induced by insulin and inhibited by LY294002 (Fig. 2), as Akt is known to be activated by insulin and inhibited by LY294002.

Activation of Akt induces cytoplasmic localization of $p21^{Cip1/WAF1}$. We next tested whether the activation of Akt affects the cellular localization of $p21^{Cip1/WAF1}$. We co-transfected $p21^{-/-}$ mouse embryonic fibroblasts (MEFs) with CA-Akt or DN-Akt together with wild-type or mutant $p21^{Cip1/WAF1}$ (the mutants containing T145A or T145D mutations) and examined the cellular localization of $p21^{Cip1/WAF1}$ by immunofluorescence analysis. As shown in Fig. 4, wild-type $p21^{Cip1/WAF1}$ was predominantly present in the cytoplasm in the presence of CA-Akt, but was predominantly in the nucleus when DN-Akt was introduced. Mutation at T145A abolished cytoplasmic localization even in the presence of CA-Akt. $p21^{Cip1/WAF1}$(T145D), which mimics $p21^{Cip1/WAF1}$ phosphorylated by Akt, was predominantly present in the cytoplasm, regardless of the activation status of the Akt pathway. These results indicate that T145 in the NLS of $p21^{Cip1/WAF1}$ is critical in determining its cellular localization, and that phosphorylation at this site by Akt results in

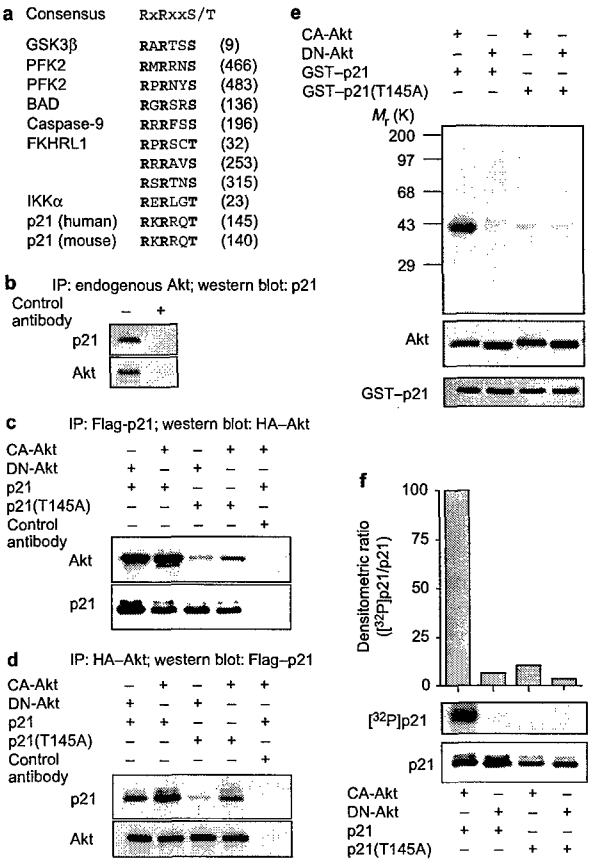

Figure 3 **Akt interacts with $p21^{Cip1/WAF1}$ and phosphorylates it at threonine 145. a**, Comparison of the amino-acid sequences of the Akt-phosphorylation motifs of $p21^{Cip1/WAF1}$ and other known Akt substrates. The consensus sequence is shown at the top. Numbers on the right indicate the positions of the final residues shown in each case. **b**, Immunoprecipitation (IP) of endogenous Akt and detection of endogenous $p21^{Cip1/WAF1}$. Endogenous Akt was immunoprecipitated from 1,000 μg of MDA-MB453 cell lysate with a specific antibody against Akt or with control immunoglobulin G. After transfer to a nitrocellulose membrane, endogenous $p21^{Cip1/WAF1}$ was detected with a specific antibody against $p21^{Cip1/WAF1}$. **c**, Immunoprecipitation of $p21^{Cip1/WAF1}$ and detection of Akt. HA-tagged DN-Akt or CA-Akt (10 μg) and Flag-tagged wild-type or mutant $p21^{Cip1/WAF1}$ (10 μg) were co-transfected into 293T cells. Cells were lysed after 48 h and $p21^{Cip1/WAF1}$ was immunoprecipitated with anti-Flag antibody. After transfer to a nitrocellulose membrane, Akt was detected with anti-HA antibody. **d**, Immunoprecipitation of Akt and western blotting of $p21^{Cip1/WAF1}$. Akt was immunoprecipitated with an anti-HA antibody and $p21^{Cip1/WAF1}$ was detected with an anti-Flag antibody. **e**, Akt phosphorylates $p21^{Cip1/WAF1}$ at T145. HA-tagged CA-Akt or DN-Akt (20 μg) was transiently transfected into 293T cells as described above. After 48 h of incubation, CA-Akt or DN-Akt was immunoprecipitated with an anti-HA antibody and incubated with 5 μg of either GST–$p21^{Cip1/WAF1}$ or GST–$p21^{Cip1/WAF1}$(T145A) in kinase buffer containing 5 μCi [γ-^{32}P]ATP for 30 min at 30 °C. The kinase reaction was terminated with SDS–PAGE buffer, and samples were assayed by autoradiography. The lower two panels show western blots for Akt and the GST-fusion protein used in the phosphorylation reaction, detected with antibodies against Akt and $p21^{Cip1/WAF1}$, respectively. **f**, CA-Akt or DN-Akt (18 μg) and Flag-tagged wild-type or mutant $p21^{Cip1/WAF1}$ (T145A mutation; 2 μg) were co-transfected into 293T cells. After 48 h cells were labelled with 1 mCi ml^{-1} [^{32}P]-orthophosphate for 3 h. $p21^{Cip1/WAF1}$ was immunoprecipitaed from lysates and analysed either by autoradiography, to detect phosphorylation of $p21^{Cip1/WAF1}$ *in vivo* (upper panel), or by western blotting, as a control for equal amounts of $p21^{Cip1/WAF1}$ (lower panel). The histogram shows the amounts of labelled $p21^{Cip1/WAF1}$ relative to the amount of immunoprecipitated $p21^{Cip1/WAF1}$ in western blots.

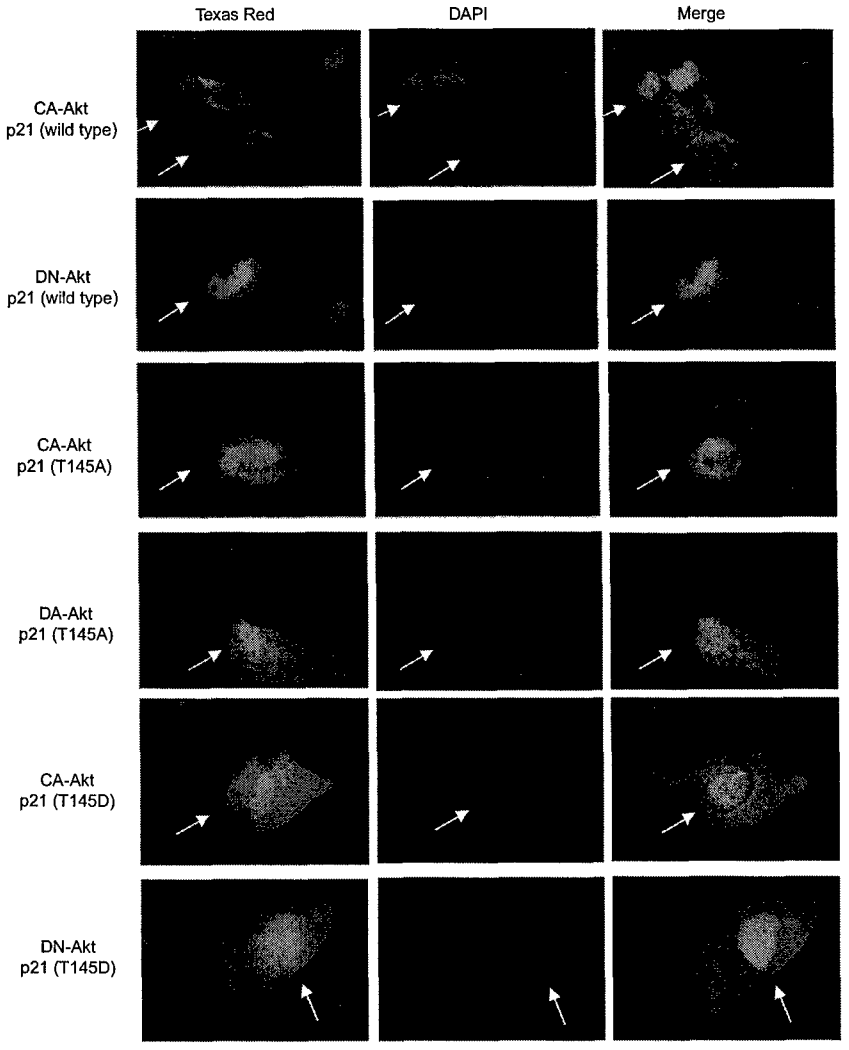

Figure 4 **Akt alters the cellular localization of p21$^{Cip1/WAF1}$.** CA-Akt or DN-Akt (9 μg) and wild-type or mutant p21$^{Cip1/WAF1}$ (1 μg) were co-transfected into p21$^{-/-}$ MEFs. After 36 h of incubation, cells were trypsinized and plated onto chamber slides for a further 12 h. After fixation, the cellular localization of p21$^{Cip1/WAF1}$ was detected using a monoclonal antibody against human p21$^{Cip1/WAF1}$. After extensive washing in PBS, samples were further incubated with Texas Red-conjugated goat anti-mouse immunoglobulin G plus DAPI and examined by fluorescence microscopy. When wild-type p21$^{Cip1/WAF1}$ was co-transfected with CA-Akt, ~75% was localized in the cytoplasm. However, when wild-type p21$^{Cip1/WAF1}$ was co-transfected with DN-Akt, ~90% was detected in the nucleus. When p21$^{Cip1/WAF1}$(T145A) was co-transfected with CA-Akt or DN-Akt, ~80% and ~90%, respectively, was localized in the nucleus. When p21$^{Cip1/WAF1}$(T145D) was cotransfected with CA-Akt or DN-Akt, ~72% and ~78%, respectively, was localized in the cytoplasm.

cytoplasmic localization of p21$^{Cip1/WAF1}$.

We carried out the above experiments in cells that were transiently transfected with exogenous genes. To investigate whether endogenous p21$^{Cip1/WAF1}$ can be regulated in a similar way, we first carried out biochemical cellular fractionation to determine the localization of endogenous p21$^{Cip1/WAF1}$ in *HER-2/neu*-overexpressing cells and their DN-Akt transfectants. p21$^{Cip1/WAF1}$ was predominantly located in the cytoplasm in both *HER-2/neu* 3T3 cells and MDA-MB453 cells, in which Akt was constitutively activated (Fig. 5a). However, when the Akt pathway was blocked by DN-Akt, p21$^{Cip1/WAF1}$ was primarily present in the nucleus in both DN-Akt 3T3 and DN-Akt/MB453 cells (Fig. 5a). As a control, we used actin and proliferating-cell nuclear antigen (PCNA) as cytoplasmic and nuclear markers, respectively, to confirm that the observed cellular localization of p21$^{Cip1/WAF1}$ was not due to contamination. Similar results were obtained when we stained *HER-2/neu* 3T3 cells and their DN-Akt transfectants for endogenous p21$^{Cip1/WAF1}$ using the same techniques as described in Fig. 4 (data not shown). To investigate further whether phosphorylation of endogenous p21$^{Cip1/WAF1}$ by Akt affects its subcellular localization, we treated MDA-MB453 cells with insulin, which is known to be a potent Akt activator (Fig. 2b). Endogenous p21$^{Cip1/WAF1}$ was present in both the nucleus and cytoplasm of MDA-MB453 cells, and the cytoplasmic fraction of p21$^{Cip1/WAF1}$ was significantly increased upon stimulation of insulin (Fig. 5b). Although equal amounts of protein were loaded onto the gel, the ratio of the protein contents of cytoplasm and nucleus was roughly 10:1 in the cellular fraction. In contrast to parental cells, endogenous p21$^{Cip1/WAF1}$ was mainly in the nucleus in DN-Akt

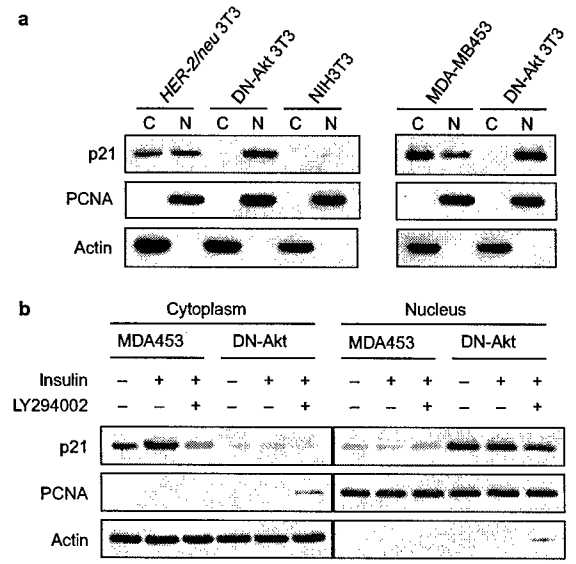

Figure 5 **Cellular localization of endogenous p21$^{Cip1/WAF1}$.** **a**, Cellular fractionation was carried out to determine the cellular localization of p21$^{Cip1/WAF1}$ in *HER-2/neu* 3T3 cells, MDA-MB453 cells, and their DN-Akt transfectants (see Methods). Equal amounts (40 μg) of cytoplasmic fraction (C) and nuclear fraction (N) from each sample were analysed by 12% SDS–PAGE. Actin and proliferating-cell nuclear antigen (PCNA) were used as markers of the cytoplasmic and nuclear fractions, respectively. In each cellular fractionation, ten times as much protein was present in the cytoplasm as in the nucleus. **b**, The cellular location of endogenous p21$^{Cip1/WAF1}$ is regulated by activation of Akt. MDA-MB453 cells were stimulated with insulin in the presence or absence of the PI-3K inhibitor LY294002. Cellular fractionation was carried out (see Methods). Equal amounts of cytoplasmic and nuclear fraction from each sample were analysed by western blotting.

transfectants, and this nuclear localization was not affected by insulin treatment. Together, these results indicate that the distribution of endogenous p21$^{Cip1/WAF1}$ can be regulated by extracellular stimuli, such as insulin, through the PI-3K/Akt pathway.

These results clearly demonstrate that p21$^{Cip1/WAF1}$ is regulated by the *HER-2/neu*–Akt pathway in cell culture. To determine whether this is also the case in tumour tissues, we compared the levels of activated (phosphorylated) Akt and the cellular localization of p21$^{Cip1/WAF1}$ in five *HER-2/neu*-positive and five *HER-2/neu*-negative human breast tumours by immunostaining with specific antibodies against phosphorylated Akt and against p21$^{Cip1/WAF1}$. Consistent with our previous results[4], Akt was activated in all *HER-2/neu*-positive breast tumours. In these tissues, p21$^{Cip1/WAF1}$ was present in both the nucleus and the cytoplasm. In contrast, in all *HER-2/neu*-negative breast tumours examined, Akt was not activated, and p21$^{Cip1/WAF1}$ was primarily localized in the nucleus. A representative experiment is shown in Fig. 6. The tumour-staining data support our findings in cell-culture experiments and further strengthen the hypothesis that overexpression of *HER-2/neu* can regulate the cellular distribution of p21$^{Cip1/WAF1}$ through activation of Akt.

Nuclear p21$^{Cip1/WAF1}$(T145A) preferentially supresses growth of transforming cells. Because Akt can phosphorylate p21$^{Cip1/WAF1}$ and cause it to be localized in the cytoplasm, we next investigated whether the phosphorylation status of p21$^{Cip1/WAF1}$ at T145 affects the cell-growth-inhibiting activity of p21$^{Cip1/WAF1}$. We transfected NIH3T3, HER-2/*neu* 3T3, and DN-Akt 3T3 cells with wild-type p21$^{Cip1/WAF1}$ and its mutants T145A (which cannot be phosphorylated) and T145D (which mimics the phosphorylated wild type), and measured their growth-inhibiting activity using a colony-formation assay. As shown in Fig. 7a, wild-type p21$^{Cip1/WAF1}$ did not effectively inhibit the growth of *HER-2/neu* 3T3 cells, in which Akt is constitutively activated, compared with NIH3T3 and DN-Akt 3T3 cells. However, p21$^{Cip1/WAF1}$(T145A) caused similar inhibition of growth in these three cell lines, and this activity was independent of activation of Akt. In contrast, p21$^{Cip1/WAF1}$(T145D) in all three cell lines behaved similarly to the wild type in *HER-2/neu* 3T3 cells. When the rate of DNA synthesis was measured by incorporation of bromodeoxyuridine (BrdU) (Fig. 7b), inhibition of growth by

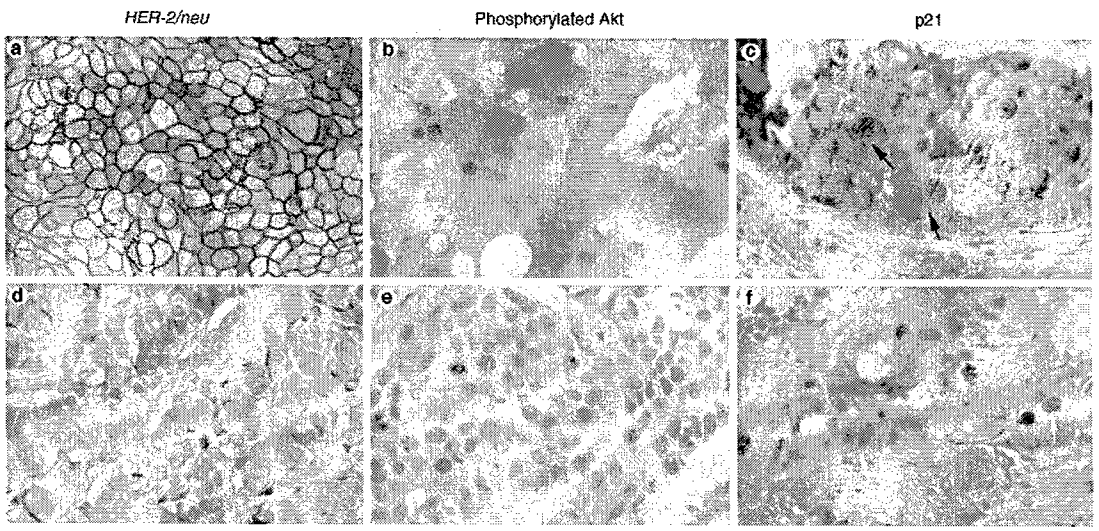

Figure 6 ***HER-2/neu* activates Akt and induces cytoplasmic localization of p21$^{Cip1/WAF1}$ in breast tumours.** Tissue sections from *HER-2/neu*-positive (**a–c**) and HER-2/*neu*-negative (**d–f**) adenocarcinomas were stained with specific antibodies against *HER-2/neu* (**a**, **d**), phosphorylated Akt (**b**, **e**) and p21$^{Cip1/WAF1}$ (**c**, **f**), or with normal rabbit serum (data not shown). Immunostaining was visualized with peroxidase-conjugated secondary antibody. Black and white arrows indicate the cytoplasmic and nuclear localization of p21$^{Cip1/WAF1}$ in **c** and **f**, respectively.

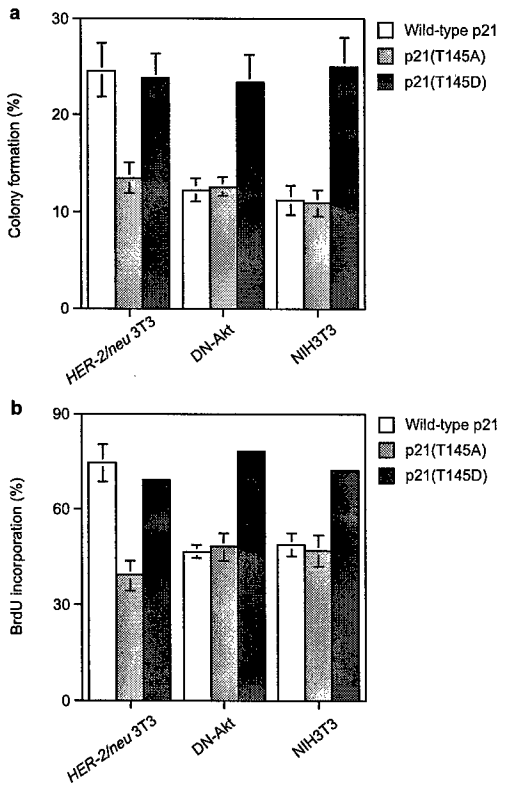

Figure 7 **Growth-inhibiting activities of p21$^{Cip1/WAF1}$ mutants.** p21$^{Cip1/WAF1}$(T145D) has reduced inhibition activity, whereas p21$^{Cip1/WAF1}$(T145A) retains its inhibition activity independently of Akt activation. **a**, The colony-formation assay was used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutants. Wild-type or mutant p21$^{Cip1/WAF1}$ (T145A or T145D mutation) or the vector pcDNA3 (2 μg of each) was transfected into NIH3T3, *HER-2/neu* 3T3, and DN-Akt 3T3 cells. The number of colonies from each transfectant was determined by crystal-violet staining. Percentages of colonies for wild-type and mutant p21$^{Cip1/WAF1}$ were calculated by defining the number obtained from vector transfection alone as 100%. Data are means ± s.e.m. from four independent experiments. **b**, The BrdU-incorporation assay was also used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutants. Vector containing membrane-bound GFP (1 μg) and wild-type or mutant p21$^{Cip1/WAF1}$ or vector pcDNA3 (9 μg each) were co-transfected into NIH3T3, *HER-2/neu* 3T3, and DN-Akt 3T3 cells, using liposome. After 48 h of incubation, cells were labelled with BrdU for 1 h and fixed with 70% ethanol. Cells were then stained with anti-BrdU antibody and incubated with fluorescein-conjugated secondary antibody. After extensive washing, cells were sorted for the presence of GFP signal, and incorporation of BrdU was measured by FACS analysis. Percentages of BrdU incorporation for wild-type and mutant p21$^{Cip1/WAF1}$ were calculated by defining the number obtained from vector (pcDNA3) alone as 100%. Data are means ± s.e.m. from two separate experiments.

wild-type p21$^{Cip1/WAF1}$ was regulated by the activation status of Akt, which is consistent with the results of the colony-formation assay. p21$^{Cip1/WAF1}$(T145A) inhibited growth independently of Akt activation. The activity of p21$^{Cip1/WAF1}$(T145D) was also independent of Akt activation, although its inhibitory effect was weaker in all three cells and was comparable to that of wild-type p21$^{Cip1/WAF1}$ in *HER-2/neu*3T3 cells, in which p21$^{Cip1/WAF1}$ is predominantly located in the cytoplasm. Why p21$^{Cip1/WAF1}$(T145D) only partially lost its ability to inhibit cell growth is not clear. One explanation is that it may still retain its ability to inhibit the function of Cdk2, Cdk4 and cyclin D,

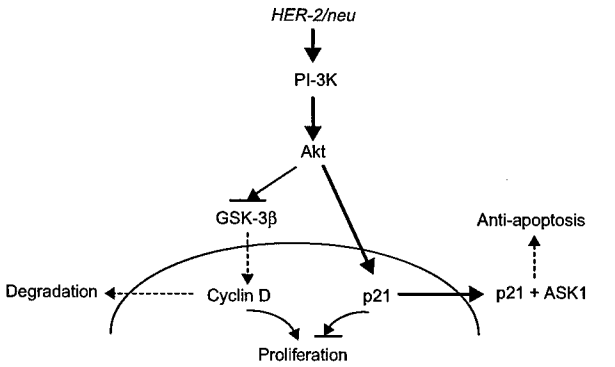

Figure 8 **Model for *HER-2/neu*-induced cytoplasmic localization of p21$^{Cip1/WAF1}$ through activation of Akt.** Dashed lines are derived from previous studies[23,28]. Solid lines are derived from the study reported here.

which shuttle between the nucleus and cytoplasm. Alternatively, cytoplasmic p21$^{Cip1/WAF1}$ may also have effects on cell growth. Further systemic study is required to elucidate the detailed mechanism. Together, our results indicate that phosphorylation at T145 in the NLS of p21$^{Cip1/WAF1}$ by Akt triggers cellular localization and thereby regulates the growth-inhibiting activity of p21$^{Cip1/WAF1}$.

Discussion

The cellular localization of p21$^{Cip1/WAF1}$ was recently proposed to be critical for the regulation of p21$^{Cip1/WAF1}$ function[27]. However, the mechanism by which the cellular localization of p21$^{Cip1/WAF1}$ is controlled was not known. Here we have identified this mechanism as phosphorylation by Akt of p21$^{Cip1/WAF1}$ at T145, which results in cytoplasmic localization and suppression of growth-inhibiting activity. Our results, together with previous findings[28], lead us to propose a plausible model for how Akt simultaneously coordinates two functionally different proteins to achieve a harmonious effect on cell proliferation (Fig. 8). On one hand, activation of Akt inhibits glycogen synthase kinase-3β (GSK-3β) and stabilizes the growth-promoting factor cyclin D in the nucleus to stimulate cell growth[28], whereas on the other hand, Akt can also phosphorylate p21$^{Cip1/WAF1}$ and cause it to localize to the cytoplasm, thereby suppressing its growth-inhibiting activity. Furthermore, our results, together other previous findings[23] also indicate a possible mechanism for the anti-apoptotic effect of p21$^{Cip1/WAF1}$. It has been shown that cytoplasmic p21$^{Cip1/WAF1}$ forms a complex with ASK1 to inhibit the stress-induced MAP kinase cascade, which results in resistance to apoptosis induced by many stimuli[23]. Overexpression of *HER-2/neu* is known to activate the Akt pathway and to confer resistance to apoptosis induced by various stimuli[4,24]. It will be interesting to determine whether this resistance is due to the formation of complexes between cytoplasmic p21$^{Cip1/WAF1}$ and ASK1 in *HER-2/neu*-overexpressing cells. In general, protein translocation is recognized as a crucial mechanism for the regulation of protein function[27]. Our results provide a clear example of regulation by Akt of the cellular location of p21$^{Cip1/WAF1}$ by phosphorylation at T145, and indicate that this process may be involved in *HER-2/neu*- or Akt-mediated cell proliferation. □

Methods

Materials.

The PI-3K inhibitor wortmannin and the DNA dye DAPI were from Roche. Anti-Flag and anti-haemagglutinin (HA; 12C5) antibodies were from Sigma and Roche, respectively. DNA plasmids encoding CA-Akt and DN-Akt were provided by P. Tsichlis (Fox Chase Cancer Center). Antibodies against Akt and p21$^{Cip1/WAF1}$ were from New England Biolabs and Santa Cruz, respectively.

p21$^{Cip1/WAF1}$ constructs.

A *Bam*HI site and an *Eco*RI site were generated by polymerase chain reaction (PCR) near the start and termination codons, respectively, in human wild-type p21$^{Cip1/WAF1}$ and were subcloned into the expression vector pcDNA3. Site-directed mutagenesis was carried out according to the manufacturer's protocol (Clontech). T145 in p21$^{Cip1/WAF1}$ was replaced with either alanine or aspartic acid, using the following primers: T145A, 5′-CGAAAACGGCGGCAGGCCAGCATGAC; T145D, 5′-CGAAAACGGCGG-CAGGACAGCATGAC. For the colony-formation assay in DN-Akt 3T3 cells, a *Bam*HI–*Eco*RI fragment containing wild-type or mutant p21$^{Cip1/WAF1}$ was subcloned into the expression vector pcDNA3–hygromycin. The sequences of the p21$^{Cip1/WAF1}$ constructs were verified by automated sequencing. To generate constructs for bacterial expression of wild-type and mutant p21$^{Cip1/WAF1}$ tagged with glutathione-*S*-transferase (GST), the same fragments were subcloned into the bacterial expression vector pGEX4T-3 (Pharmacia). Wild-type and mutant p21$^{Cip1/WAF1}$ proteins were inducibly expressed in *Escherichia coli* strain BL21 and purified by glutathione–sepharose chromatography (Pharmacia).

Cell culture.

NIH3T3, HER-2/*neu* 3T3 and MDA-MB453 cells, p21$^{Cip1/WAF1}$-deficient MEFs, and 293T cells were cultured in DMEM/F12 supplemented with 10% FBS. DN-Akt transfectants of *HER-2/neu* 3T3 and MDA-MB453 cells were grown under the same conditions, except that 450 μg ml^{-1} G418 was added to the culture medium[4]. 293T cells were transfected using the calcium-phosphate technique and p21$^{Cip1/WAF1}$-deficient cells by the liposome method (Cell and Molecular Technologies, Lavallette, New Jersey).

In vitro growth-rate analysis.

In vitro growth rates of the cell lines were assessed by counting cells with a Coulter counter or by the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenylterazolium bromide) assay as described[29].

[^{3}H]thymidine-incorporation assay.

Proliferation rates of the cell lines were analysed by measuring incorporation of [^{3}H]thymidine as described[29].

Endoproteinase cleavage and two-dimensional phosphopeptide analysis.

Two-dimensional analysis of p21$^{Cip1/WAF1}$ phosphopeptides was carried out using a HTLE-7000 electrophoresis system (CBS Scientific, Del Mar, California) as described[30]. Briefly, MDA-MB453 cells were incubated with 1.5 mCi ml^{-1} [^{32}P]orthophosphate for 3.5 h and then stimulated for 30 min with insulin in the presence or absence of LY294002. Endogenous p21$^{Cip1/WAF1}$ was immunoprecipitated, blotted, and visualized by autoradiography. The ^{32}P-labelled p21$^{Cip1/WAF1}$ protein on the nitrocellulose membrane was excised and digested with Tosyl-L-phenylalanine chloromethyl ketone (TPCK)–trypsin (Sigma). Completely digested phosphopeptides were spotted onto thin-layer cellulose plates (20 × 20 cm) and separated in the first dimension by electrophoresis at 1,000 V for 35 min in buffer (2.2% formic acid and 7.8% acetic acid, pH 1.9). Cellulose plates were then placed in a chromatography tank containing phosphochromatography buffer (38% *N*-butanol, 25% pyridine and 7.5% acetic acid) for 6–8 h to separate the phosphopeptides in the second dimension. Dried cellulose plates were then exposed to Kodak X-AR film.

Edman degradation.

Modified manual Edman degradation was carried out as described[31]. Briefly, phosphopeptides were covalently coupled to Sequelon-AA discs (Perseptive Biosystem Inc., Foster City, California) and subjected to consecutive cycles of Edman degradation. After each cycle, discs were treated with trifluoroacetic acid to cleave and release the amino-terminal amino acid, and the amount of radioisotope released was measured by Cerenkov counting.

In vitro kinase assay.

293T cells (0.2 × 10^6) were transfected with 20 μg of HA-tagged CA-Akt or DN-Akt. After 48 h, Akt was immunoprecipitated from cell extracts and incubated with 5 μg of purified GST–p21$^{Cip1/WAF1}$ (wild-type or mutant) in the presence of 5 μCi [γ-^{32}P]ATP and 50 mM cold ATP in kinase buffer[9] for 30 min at 30 °C. Reaction products were resolved by SDS–PAGE, and ^{32}P-labelled proteins were visualized by autoradiography.

[^{32}P]orthophosphate labelling.

293T cells (0.2 × 10^6) were co-transfected with 18 μg of CA-Akt or DN-Akt and 2 μg of wild-type or mutant p21$^{Cip1/WAF1}$. After 36 h, cells were starved for 12 h and then incubated in phosphate-free medium for 1 h. Cells were then labelled with 1 mCi ml^{-1} [^{32}P]orthophosphate for 3 h. They were then lysed, and p21$^{Cip1/WAF1}$ was immunoprecipitated from cell extracts and separated by 12% SDS–PAGE. Incorporation of ^{32}P-phosphate was measured by autoradiography.

Immunoprecipitation and immunoblotting.

Cells were washed twice with PBS and scraped into 500 μl of lysis buffer. After brief sonication, the lysate was centrifuged at 14,000*g* for 10 min at 4 °C to remove insoluble cell debris. Immunoprecipitation and immunoblotting were carried out as described[4].

In situ immunofluorescent staining.

Roughly 0.2 × 10^6 p21$^{-/-}$ MEFs were plated in 100-mm plates and co-transfected with 9 μg of CA-Akt or DN-Akt and 1 μg of wild-type or mutant p21$^{Cip1/WAF1}$, using liposome. After 36 h of incubation, cells were trypsinized and plated onto chamber slides for a further 12 h. After fixation of samples in acetone for 10 min at 4 °C, the cellular localization of p21$^{Cip1/WAF1}$ was determined using a monoclonal antibody against human p21$^{Cip1/WAF1}$ (1:100; Santa Cruz). After extensive washing in PBS, samples were further incubated with Texas Red-conjugated goat anti-mouse immunoglobulin G (1:400) and DAPI (0.1 μg ml^{-1}) for 1 h. After extensive washing, samples were examined by fluorescence microscopy (Zeiss). The nonspecific reaction of secondary antibody was ruled out by the absence of fluorescence under the microscope.

Cellular fractionation.

Roughly 2 × 10^7 cells were pelleted and resuspended in 800 μl of buffer A (10 mM HEPES pH 7.4, 1 mM EDTA and 1 mM dithiothreitol) containing the protease inhibitors phenylmethylsulphonyl fluoride, leupeptin, aprotinin and pepstatin. After incubation on ice for 10 min, cells were homogenized with ten strokes in a Dounce homogenizer. They were then examined under the microscope to confirm that >98% of cells were lysed. After brief centrifugation at 4 °C, the supernatant (cytoplasmic fraction) was collected and the pellet was washed twice with 400 μl of buffer B[23] and then resuspended in 150 μl of buffer C[23] with gentle rocking for 30 min at 4 °C (ref. 23). After centrifugation, the supernatant (nuclear fraction) was collected. The amounts of protein in the cytoplasmic and nuclear fractions were determined with a protein assay kit (Bio-Rad) and the protein was subjected to immunoblotting.

Colony-formation assay.

The colony-formation assay was used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutants. Wild-type or mutant p21$^{Cip1/WAF1}$ (T145A or T145D mutation) or the vector pcDNA3 (2 μg of each) was transfected into NIH3T3, *HER-2/neu* 3T3 and DN-Akt 3T3 cells in six-well plates, using liposome. After 48 h, cells were trypsinized and evenly distributed into four 100-mm culture plates. Cells were selected with 700 μg ml^{-1} G418 (or 100 μg ml^{-1} hygromycin for DN-Akt 3T3 cells, as they contain the neomycin-resistance gene) for 3 weeks.

Bromodeoxyuridine (BrdU) incorporation.

The BrdU-incorporation assay was also used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutants. Vector containing membrane-bound green fluorescent protein (GFP, 1 μg) and wild-type or mutant p21$^{Cip1/WAF1}$ (T145A or T145D mutation) or the vector pcDNA3 (9 μg of each) were co-transfected into NIH3T3, *HER-2/neu* 3T3 and DN-Akt 3T3 cells, using liposome. After 48 h, cells were labelled with BrdU for 1 h and fixed with 70% ethanol. Cells were then stained with anti-BrdU antibody and incubated with fluorescein-conjugated secondary antibody. After extensive washing, cells were sorted for GFP signal, and incorporation of BrdU was measured by FACS analysis.

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Ceramide does not act as a general second messenger for ultraviolet-induced apoptosis

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Ceramide has been proposed as a second messenger for stress-induced apoptosis. By characterization of murine melanoma cells and their E1A transfectants, we found several lines of evidences against the role of ceramide as a second messenger for ultraviolet (UV)-induced apoptosis. First, although E1A transfected melanoma cells were more sensitive to UV-induced apoptosis than parental cells, the relative endogenous ceramide elevation induced by UV was greater in parental cells than in E1A transfectants. Second, UV-resistant melanoma cells were more sensitive to exogenous ceramide than UV-sensitive E1A transfectants. The differential responses to UV and ceramide by E1A require the same functional CR2 domain of E1A. Third, unlike the action of UV, transient exposure (up to 2 h) of lethal dose of ceramide was not sufficient to cause apoptosis in these cells, and persistent presence of ceramide was required for processing the apoptotic process. Finally, ceramide and UV do not share a common pathway in apoptosis induction. UV-induced apoptosis was blocked by interleukin-1β-converting enzyme (ICE) inhibitor z-VAD whereas ceramide-induced apoptosis was not. Therefore, we conclude that ceramide is not a general second messenger for UV-induced apoptosis.
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Keywords: apoptosis; UV; second messenger; ceramide; E1A

Introduction

Apoptosis is initiated by a variety of physiological stimuli and environmental stresses, followed by tightly regulated cascades that activate specific proteases and nucleases that in turn carry out an ordered disassembly of cellular structures (Martin and Green, 1995; White, 1996; Wylli, 1980). Recently, attention has been directed at mechanisms by which extracellular signals activate the interleukin-1β-converting enzyme (ICE)/ced3 proteases to effect apoptosis. One important transmembrane signaling systems that may be involved is the sphingomyelin/ceramide pathway (Haimovitz-Friedman *et al.*, 1994; Hannun, 1996; Hannun and Obeid, 1995; Kolesnick *et al.*, 1994). In this proposed model, stimulation of cell surface receptors, either by cytokines or environmental stresses, activates a plasma membrane neutral sphingomyelinase (nSMase) that hydrolyzes sphingomyelin to generate ceramide and phosphocholine (Cheng and Caffrey, 1996; Devary *et al.*, 1992). Ceramide then serves as a second messenger, initiating apoptosis through the stress-activated protein kinase (SAPK/JNK) cascade (Pena *et al.*, 1997). The sphingomyelin/ceramide pathway can be activated by various stresses (X-ray, ultraviolet (UV) radiation, tumor necrosis factor alpha (TNF-α), Fas, heat shock and H_2O_2) and initiate subsequent apoptotic cascades (Kolesnick *et al.*, 1994; Martin *et al.*, 1995; Pena *et al.*, 1997; Verheij *et al.*, 1996). Thus, the sphingomyelin/ceramide signal transduction pathway appears to play an important role in mediation of stress-induced apoptosis.

The adenovirus type 5 E1A gene has been shown to sensitize cells to apoptosis induced by various stresses (Deng *et al.*, 1998; Lowe and Ruley, 1993; Mymryk *et al.*, 1994; Sabbatini *et al.*, 1995), which provides a unique tool to study the mechanism of apoptosis induction. Previously, we have shown that E1A-transfected cancer cells have increased sensitivity to apoptosis induced by various stresses and cytokines (Deng *et al.*, 1998; Shao *et al.*, 1997, 1998). Generally, E1A-expressing cells do not undergo spontaneous apoptosis; they do so only when additional stress signals are applied (Lowe and Ruley, 1993; Mymryk *et al.*, 1994; Sabbatini *et al.*, 1995; Deng *et al.*, 1998). This implies that altered stress signal transduction might play a role in initiation of apoptosis in E1A-expressing cells. Since the ceramide pathway has been shown to play an important role in stress-induced apoptosis, we asked whether the sphingomyelin/ceramide pathway is involved in E1A-mediated apoptosis in response to stresses. In this study, we characterized the possible role of ceramide in UV-induced apoptosis in both E1A transfectants and parental cells. Unexpectedly, we found that ceramide is unlikely to be a second messenger for UV-induced apoptosis.

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Results

Apoptotic process in E1A transfectants was initiated within 1 h following UV exposure

To determine the effect of E1A on cellular response to environmental stresses, we examined the susceptibility of murine melanoma cells, K1735 M2, and stable E1A transfectants of these cells (Deng *et al.*, 1998) to UV irradiation. This cell line was a metastatic variant derived from K1735 melanoma cells (Pierceall *et al.*, 1992). Both parental tumor cells (K) and vector plasmid (pSV-neo) transfectants (KSP) were resistant to UV, whereas the E1A transfectants (KA4 and KA9) were sensitive to UV-induced apoptosis (Figure 1a). The effect of E1A-mediated sensitization was dramatic: UV at a low dose (5 J/m^2) induced significant apoptosis in KA9 cells within 24 h, whereas UV at more than 100 times that dose (600 J/m^2) had only minimal effect on parental K cells (Figure 1b). For convenience of description, the results from the KA4 and KSP cells in the following studies are not shown although they behave similarly to KA9 and K cells, respectively. We next examined the kinetics of UV-induced apoptosis in E1A transfectants. The E1A-mediated apoptotic response to UV was rapid, and fragmented DNA appeared as early as 4 h after exposure (Figure 1c). To determine the onset of biochemical process of apoptosis, we examined PARP cleavage. PARP is a DNA repair enzyme that contributes to genomic integrity and provides a critical defense against cellular stress. PARP (113 kD) is inactivated and cleaved into 85 kD fragment at the onset of apoptosis by proteases; this inactivation precedes DNA fragmentation (Bose *et al.*, 1995). UV irradiation produced no cleavage of PARP in parental cells and the PARP expression level in these cells was increased at 4 h, presumably reflecting DNA repair. However, in E1A transfectants, PARP cleavage began as early as 1 h after UV exposure and was completed by 4 h. This observation suggests that initiation of the apoptotic cascade in E1A transfectants by UV is a quick process that precedes this time point (1 h) (Chen *et al.*, 1996).

Ceramide is not involved in the E1A-mediated sensitization to UV-induced apoptosis

Because ceramide is a proposed second messenger for UV-induced apoptosis (Pena *et al.*, 1997), we asked whether the ceramide pathway is involved in E1A-mediated sensitization to UV. If so, E1A transfected cells might have either an increased ability to generate ceramide in response to UV or an increased sensitivity to ceramide. To determine whether E1A transfectants

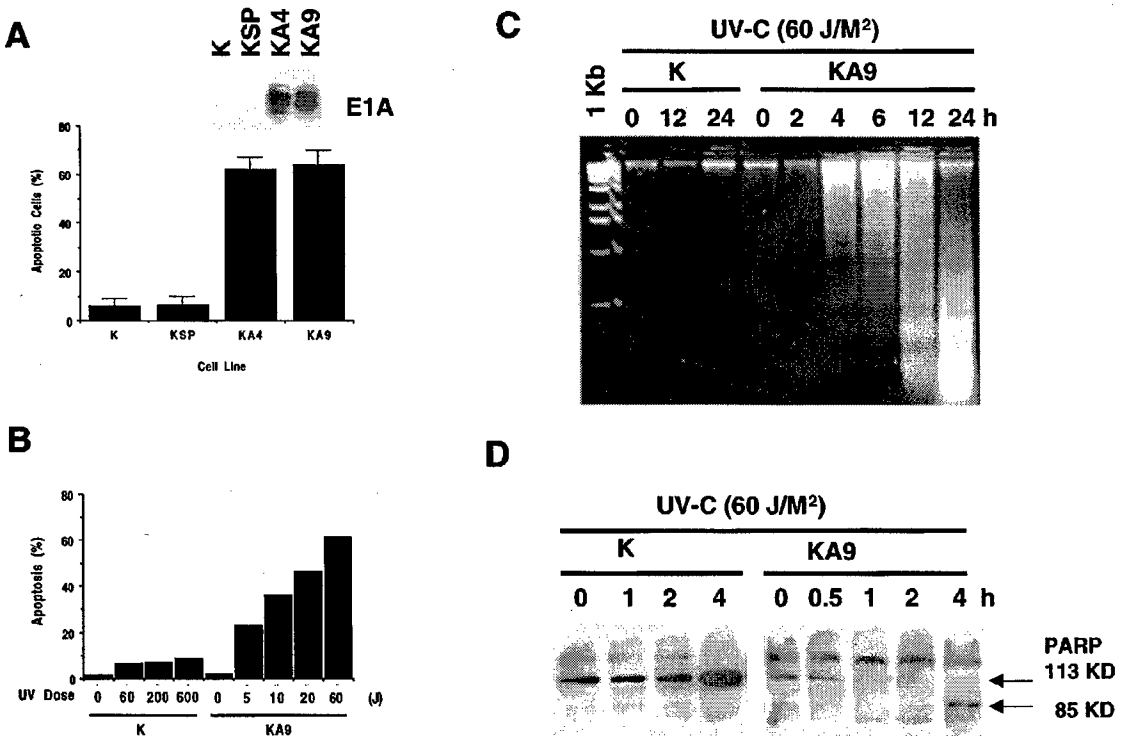

Figure 1 E1A-mediated sensitization to UV irradiation. (**a**) Induction of apoptosis (as assessed by subdiploid cell population) by UV irradiation (60 J/m^2 for 24 h) in murine melanoma cells (K), neomycin plasmid transfectants (KSP) and E1A transfectants (KA4 and KA9). The insert at the top is an immunoblot against E1A gene products. (**b**) Induction of apoptosis in K and KA9 cells by UV irradiation (24 h) at the indicated doses. (**c**) Kinetics of UV (60 J/m^2)-induced DNA fragmentation in K and KA9 cells. Similar results were obtained in KSP and KA4 cells (not shown). (**d**) Kinetics of PARP cleavage in K and KA9 cells after UV irradiation. PARP expression level was determined by immunoblot with antibody against PARP

have an increased ability to generate ceramide, we analysed the endogenous ceramide levels in both E1A transfectants and parental cells following UV irradiation. Interestingly, the magnitude of relative ceramide increase after UV irradiation was greater in UV-resistant parental cells than in UV-sensitive E1A transfectants (Figure 2a). This observation suggests that E1A-mediated sensitization does not take place at a level through an increased generation of ceramide in response to UV irradiation if ceramide functions as a second messenger. Next, we addressed whether E1A transfectants had an increased sensitivity to ceramide by examining the sensitivity of both E1A transfectants and parental cells to exogenous C2-ceramide. Unexpectedly, we found that E1A transfectants were more resistant, rather than sensitive, to ceramide than parental tumor cells (Figure 2b). Similar results were obtained by treatment of these cells with C6-ceramide (data not shown). Since E1A transfectants had neither an increased ability to generate ceramide nor an increased sensitivity to ceramide, we conclude that ceramide unlikely plays a role in E1A-mediated sensitization to UV-induced apoptosis. Moreover, characteristic features of UV-induced endogenous ceramide elevation and ceramide-induced apoptosis in E1A, transfectants and parental cells were inconsistent with the expected role of ceramide as a second messenger for UV-induced apoptosis.

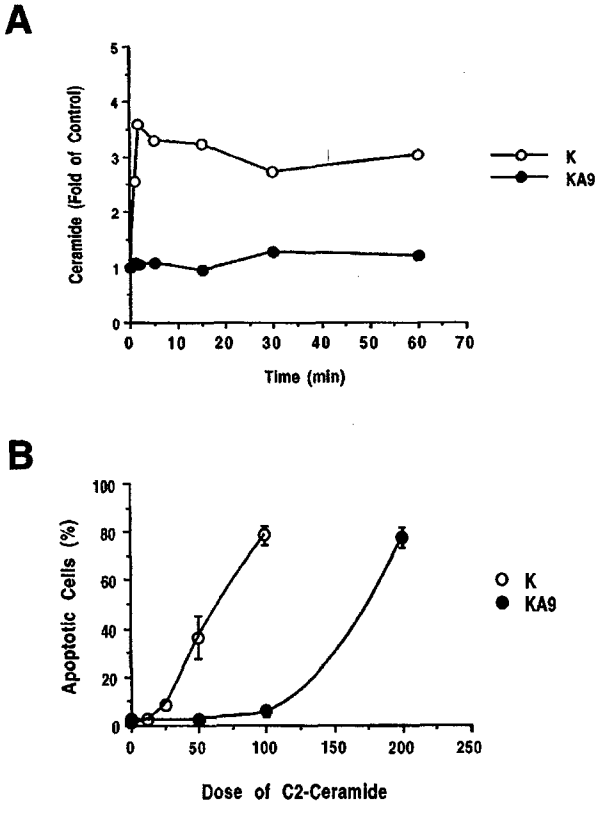

Figure 2 Comparison of ceramide generation by UV and sensitivity to ceramide in the parental murine melanoma cell line and its E1A transfectants. (**a**) Kinetics of relative endogenous ceramide levels in both E1A transfectants (KA9) and parental cells (K) were analysed at the indicated times after UV irradiation (60 J/m^2). (**b**) Induction of apoptosis in K and KA9 cells by C2-ceramide at the indicated doses, 24 h after the treatment, apoptotic cells were harvested for FACS analysis

Differential responses to UV and ceramide by E1A require the same functional CR2 domain

The E1A gene products have multiple functions on host cells by interacting with multiple cellular regulatory proteins such as p300 (Chen *et al.*, 1996; Eckner *et al.*, 1994) and Rb (Egan *et al.*, 1989; Whyte *et al.*, 1988) through different functional domains. In an initial attempt to characterize which domains of E1A responsible for sensitization and resistance to apoptosis by UV and ceramide, respectively, we examined UV- and ceramide-induced apoptosis on mutant E1A transfectants. The mutant E1A dl1101 contains a deletion at the N-terminus that is required for interaction with p300, and the mutant E1A dl1108 contains a deletion at the CR2 domain that is required for interaction with Rb (Egan *et al.*, 1989; Shisler *et al.*, 1996). Both DNA fragmentation and FACS analysis showed the mutant E1A dl1101 stable transfectants, K01.9, to be sensitive to UV as were the KA9 wild type E1A transfectants; but the mutant E1A dl1108 stable transfectants, K08.5, were resistant to UV irradiation (Figure 3a,b,e). These results suggest that the CR2 domain, but not the N-terminus, of E1A is required for E1A-mediated sensitization to UV irradiation. Interestingly, the sensitivity of these mutant E1A stable transfectants to C2-ceramide (Figure 3c,e) was completely opposite that of their sensitivity to UV (Figure 3b,d), suggesting that the CR2 domain is also required for E1A-mediated resistance to ceramide. Thus, differential responses to UV and ceramide by E1A required the same functional CR2 domain of E1A. This further supports the notion that ceramide unlikely plays a role in mediation of UV-induced apoptosis in E1A transfectants.

Ceramide-induced apoptosis is kinetically different from UV-induced apoptosis

It should be noticed that the ceramide pathway in parental melanoma cells is intact, since UV induced endogenous ceramide elevation (Figure 2a) and exogenous ceramide induced apoptosis in these cells (Figure 2b). However, they were highly resistant to UV-induced apoptosis (Figure 1b). This suggests that ceramide might not act as a general second messenger for UV-induced apoptosis. According to the model of the ceramide pathway, the acute ceramide elevation induced by stresses including UV, which occurs in seconds to minutes, serves as a second messenger for apoptosis induction (Hannun, 1996; Kolesnick *et al.*, 1994; Pena *et al.*, 1997; Verheij *et al.*, 1996). If so, the apoptotic process should be inducible by a transient exposure of cells to exogenous ceramide, which mimics the kinetics of acute ceramide elevation by stresses

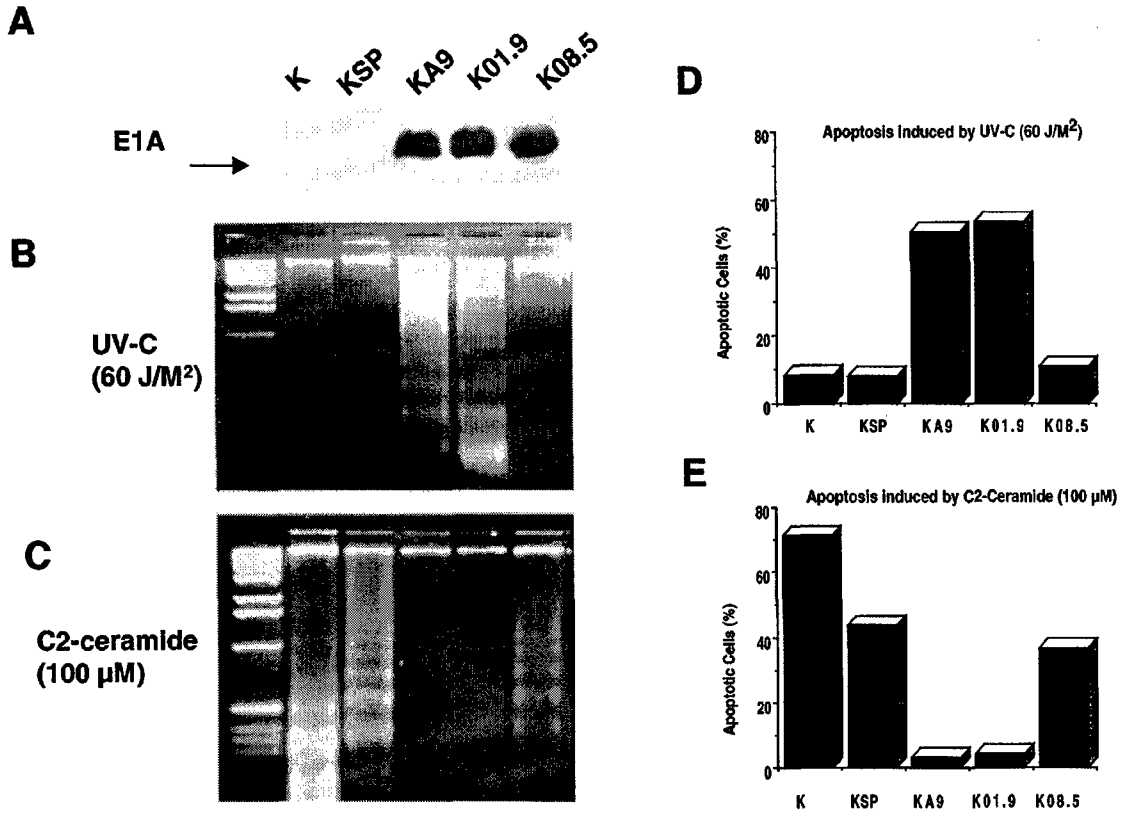

Figure 3 Different sensitivities of E1A transfectants, mutant E1A transfectants, and parental cells to ceramide and UV. (**a**) Immunoblot with antibody against the E1A gene products in different E1A and mutant E1A transfectants. K01.9 and K08.5 cells were mutant E1A dl1101 and dl1108 stable transfectants, respectively. (**b**) DNA fragmentation of various cell lines treated with UV irradiation (60 J/m^2 for 24 h). (**c**) DNA fragmentation of various cell lines treated with C2-ceramide (100 μM, 24 h). (**d**) FACS analysis of various cell lines treated with UV irradiation (60 J/m^2). (**e**) FACS analysis of various cell lines treated with C2-ceramide (100 μM for 24 h). Similar results were obtained in other clones of E1A and mutant E1A (not shown)

(Dbaibo *et al.*, 1995; McConkey *et al.*, 1996). Or, as the apoptotic process is irreversible, once the program is initiated, the presence of the initial signal is no longer required. We then examined the kinetics of ceramide-induced apoptosis and compared that with the effect of ceramide on cells by transient exposure. Treatment of cells with a lethal dose of ceramide (100 μM for K and 200 μM for KA9) induced a similar apoptotic kinetics in both parental cells and E1A transfectants, which began at about 2 h, and reached a plateau in about 6 to 12 h (Figure 2a). To determine whether ceramide can kinetically mimic the action of stress, both K and KA9 were transiently exposed to C2-ceramide for different periods, then removed off ceramide and replaced with normal growth medium for incubation up to 12 h. The results indicated that short exposures (up to 2 h) of cells to a lethal dose of ceramide did not cause significant apoptosis, whereas longer exposure time (>2 h) was required for induction of apoptosis (Figure 4b). Apoptotic process induced by stresses, such as UV, is known to be irreversible. Once the apoptotic program was initiated, such as 30 s UV exposure in the current study, presence of stress (UV) source is not required for continuing the process. Interestingly, by comparing the kinetic features, we found that the percentage of apoptotic cells were identical between exposure groups (cells exposed to ceramide for different times plus additional incubation without ceramide up to total 12 h, as 'expo.' in Figure 4c,d) and harvest group (cells exposed to ceramide for different time and harvested at that time, as 'harv.' in Figure 4c,d). This suggests that, additional incubation does not further increase the level of ceramide-induced apoptosis as soon as ceramide is removed. In other words, once ceramide is removed, the apoptotic process stops immediately. Taken together, the results of this analysis suggest that, (1) transient exposure of ceramide (up to 2 h) is not sufficient to induce apoptosis; (2) persistent presence of ceramide (>2–6 h) was required for processing ceramide-induced apoptosis; and (3) ceramide-mediated apoptosis stop as soon as ceramide is removed. Thus, ceramide-induced apoptosis, which requires the presence of ceramide, is kinetically different from UV-induced apoptosis that does not require the presence of stress-source. Thus, the kinetic feature of ceramide is inconsistent with the action of an expected second messenger.

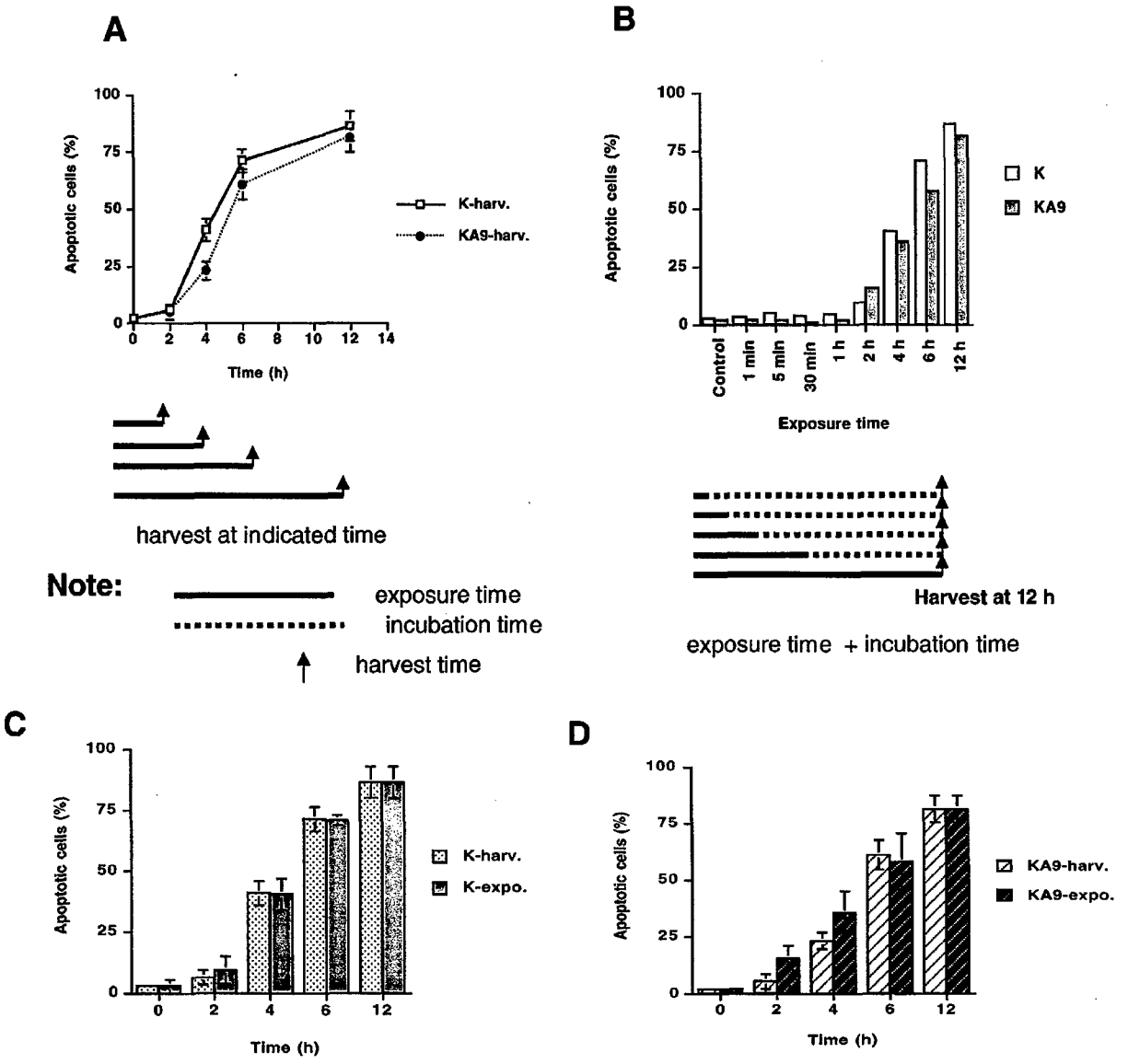

Figure 4 Kinetic analysis of ceramide-induced apoptosis. (**a**) Kinetics of ceramide-induced apoptosis. Because of different cellular sensitivities, different lethal doses of C2-ceramide were used for analysing apoptosis in K cells (100 μM C2-ceramide) and KA9 cells (200 μM C2-ceramide) (see Figure 2b). Apoptotic cells were harvested at the indicated times. (**b**) Induction of apoptosis by transient exposure of cells to C2-ceramide. Both K and KA9 cells were transiently treated with C2-ceramide (100 μM for K and 200 μM for KA9) for the indicated times. Then, the ceramide-containing medium was removed, the cells were washed once with medium and then incubated with fresh growth medium. Apoptotic cells were harvested for FACS analysis 12 h after the beginning of the treatment. (**c**) Comparison of apoptosis obtained at harvesting time ('harv.') vs exposure time ('expo.') in parental cells (K), and (**d**) in E1A transfectants (KA9). Data in **c** and **d** were obtained from the experiments in **a** and **b**. Experiments were repeated at least three times. Data are presented as mean $\pm$ s.d.

Caspases 3 is required for UV-induced but not for ceramide-induced apoptosis

Finally, we asked whether ceramide and UV share a common pathway in apoptosis induction. We assumed that UV and ceramide should share a common pathway in apoptosis induction if ceramide served as a second messenger for UV-induced apoptosis. Extracellular stress signals, including UV irradiation, have been shown to activate the ICE/ced3 proteases through signal transduction pathways to effect apoptosis (Chen *et al.*, 1996). To distinguish the biochemical pathways of UV- and ceramide-induced apoptosis, we characterized the apoptotic pathways of UV or ceramide by the caspase 3 inhibitor z-VAD. Treatment of cells with z-VAD blocked UV-induced apoptosis, but did not affect ceramide-induced apoptosis in either E1A transfectants or parental cells (Figure 5a). Similar results were also obtained in the human breast cancer cell line MDA-MB-231 and 231-E1A transfectants (Figure 5b),

suggesting there is a generality of this feature. We then examined the PARP cleavage products in UV- and ceramide-treated cells by Western blot. Using Ab#1 (C2-10, Pharmigen, San Diego, CA, USA) that recognizes both full length PARP (113 kD) and its cleaved products (85 kD) (Kaufmann *et al.*, 1993), we found that PARP was mostly cleaved as a 85 kD fragment in UV-treated cells, and this function can be blocked by z-VAD (Figure 5c, upper), which is consistent with UV-induced apoptosis (Figure 5a). On the contrary, only a trace amount of PARP 85 kD fragment was detected in ceramide-treated cells, which was also inhibited by z-VAD. However, this small portion of cleaved PARP 85 kD fragment that is sensitive to caspase inhibitor z-VAD can not explain the ceramide-induced apoptosis that is resistant to the z-VAD inhibitor (Figure 5a). The results suggest that caspase 3 may not be the key caspase for ceramide-induced apoptosis, and other mechanisms must be involved in the ceramide-induced apoptosis. In consistent to this notion, a novel PARP 105 kD fragment was detected in ceramide-treated cells (Figure 5c, middle). Using Ab#2 (New England BioLabs, Beverly, MA, USA) (that only recognizes cleaved PARP, but not full length PARP) on the same membrane, we detected a 105 kD fragment that was only present in ceramide- but not UV-treated cells (Figure 5c, middle). The appearance of this product was not affected by z-VAD. To compare the subtle difference in molecular weight of this product with full length and the 85 kD fragment of PARP, we then blotted the membrane with two antibodies (Ab#1 and Ab#2) together. We found that the 105 kD band is clearly different from both the 85 kD and the full length PARP (115 kD) (Figure 5c, bottom). From the protein sequence of PARP, we found that, in addition to the EVDG sequence at 215

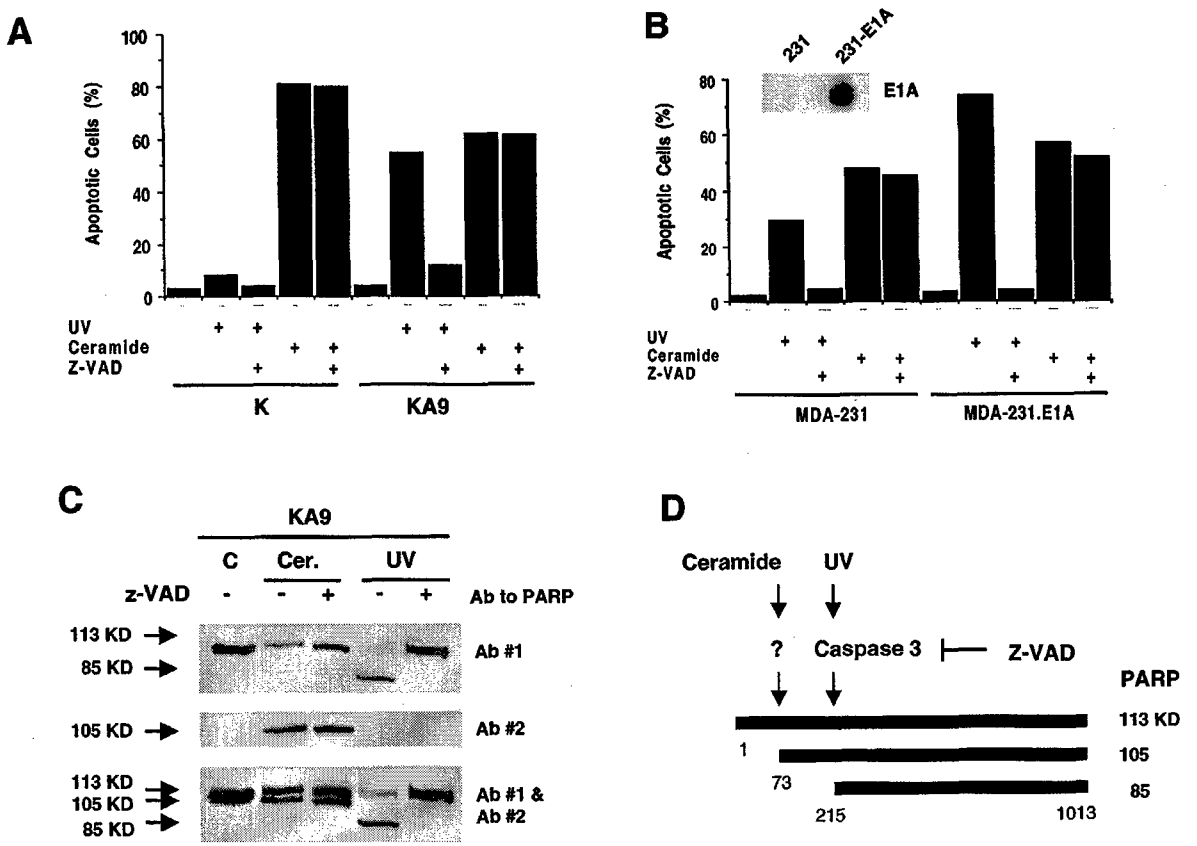

Figure 5 Ceramide- and UV-induced apoptosis go through different pathways. (**a**) The ICE inhibitor z-VAD blocked UV-induced apoptosis but not ceramide-induced apoptosis melanoma cells. K and KA9 cells were treated with UV (60 J/m^2) or ceramide (100 μM for K cells and 200 μM for KA9 cells), in the absence or presence of z-VAD (40 μM), and apoptotic cells were harvested 12 h later for FACS analysis. Similar results were also obtained for KSP and KA4 cells (not shown). (**b**) z-VAD blocked UV-induced apoptosis, but not ceramide-induced apoptosis in MAD-MB-231 human breast cancer cells, and their E1A stable transfectants. The insert at top is an immunoblot against E1A. The treatment was the same as described for **a**, except 100 μM C2-ceramide was used for both MDA-MB-231 cell line and the E1A transfectants. Similar experiments were repeated at least twice. Representative data are shown. (**c**) UV and ceramide induced different PARP cleavage. KA9 cells were treated with UV (60 J/m^2) or C2-ceramide (100 μM) in the presence or absence of 30 μM z-VAD, cells were then harvested for Western blot analysis. The membrane hybridized with the monoclonal antibody (C2-10)(Ab#1, Pharmingen) against the C-terminus of PARP (top). The membrane was stripped and re-hybridized with polyclonal antibody recognizing only the cleavage fragment of PARP (Ab#2, New England Biolabs) (middle). Then, both antibodies were combined to blot the same membrane (bottom). (**d**) The proposed model of UV- and ceramide-induced PARP cleavage. The proposed cleavage EVDG sequence is found at both 73 and 215 sites of PARP sequence

(that is cleaved by the caspase 3 between Asp and Gly), another EVDG sequence appears at the 73 site on PARP. Theoretically, cleavage of the EVDG at 73 site would result in a 105 kD product. As the combined intensity of the cleaved and uncleaved bands in ceramide-treated cells is about the same as that of the full length band in control cells (protein loading of each sample was the same) (Figure 5c, bottom), it is likely that the 105 kD band coming from cleavage at this site. Although further study is required to determine the identity of the 105 kD product and the caspase responsible for the action, the results clearly indicate that the mechanisms of UV- and ceramide-induced apoptosis are apparently different. Therefore, ceramide is unlikely to serve as a general second messenger for UV-induced apoptosis.

Discussion

The sphingomyelin/ceramide signal transduction pathway was proposed to mediate apoptosis in response to various types of stresses, including TNF-α, Fas, ionizing radiation, UV-C, X-ray, H_2O_2, and heat shock (Martin *et al.*, 1995; Verheij *et al.*, 1996). The model was mainly supported by two important lines of evidence, first that stress stimuli induce acute elevation in ceramide concentration within seconds to minutes, which precedes the cellular effects of these stimuli; and second that ectopic administration or generation of ceramide induces apoptosis, which mimics stress-induced apoptosis (Hannun, 1996; Kolesnick *et al.*, 1994; Pena *et al.*, 1997).

In this study, we examined whether the ceramide pathway play a role in UV-induced apoptosis in E1A transfectants. Unexpectedly, we found that ceramide not only does not play a role in E1A-mediated sensitization of apoptosis, but also does not serve as a general second messenger for UV-induced apoptosis. There were several evidences against the original ceramide model. First, UV-induced endogenous ceramide elevation in parental melanoma cells and E1A transfectants were inconsistent with the concept of ceramide as a second messenger (Figures 1 and 2a). Second, although E1A sensitized melanoma cells to UV-induced apoptosis, E1A also rendered these cells resistant, rather than sensitive, to ceramide-mediated apoptosis (Figures 2b and 3), suggesting that UV- and ceramide-induced apoptosis might be different. Third, the kinetics of ceramide-induced apoptosis (Figure 4) is different from that of UV-induced apoptosis, which is inconsistent with the role of an expected second messenger. Finally, caspase inhibitor z-VAD blocked the UV-mediated apoptosis and PARP cleavage, that had no effect on ceramide-induced apoptotic process. This suggests that the molecular mechanisms of UV-induced apoptosis and ceramide-induced apoptosis are different. Thus, ceramide is unlikely to serve as a second messenger for the UV-induced apoptosis.

E1A-transfected murine melanoma cells were unexpectedly found to be more resistant to exogenous ceramide-induced apoptosis than parental cells. This function, similar to the E1A-induced UV sensitivity, requires CR2 domain of E1A (Figure 3c). The CR2 domain of E1A has been shown to interact with Rb and the Rb family proteins p107 and p130 (Mymryk *et al.*, 1994; Shisler *et al.*, 1996). The Rb gene product is reported to be a downstream target for a ceramide-induced apoptosis and growth arrest (Dbaibo *et al.*, 1995; McConkey *et al.*, 1996). It remains to be seen whether interactions between E1A and Rb family proteins might play a role in the resistance and sensitization to the ceramide- and UV-induced apoptosis, respectively in the E1A-transfected melanoma cells. However, expression of E1A in MDA-MB-231 human breast cancer cells did not change the sensitivity of host cells to ceramide although E1A increased their sensitivity to UV irradiation (Figure 5b). The discrepancy between E1A effects on the murine melanoma cells and the human breast cancer cells is not clear. It may be due to different cellular context. Nevertheless, differential responses of E1A-transfected cells to UV and ceramide prompt us to re-evaluate the role of ceramide in apoptosis induction by stresses like UV. Importantly, we found that UV- and ceramide-induced apoptosis have different sensitivity to z-VAD. Moreover, the PARP cleavage patterns were different between UV- and ceramide-treated cells. Caspase 3 is known to be required for execution of apoptosis and PARP cleavage (between Asp214 and Gly215, resulting in a 85 kD fragment), which can be blocked by z-VAD. Inhibition of UV-induced apoptosis and PARP cleavage suggests that caspase 3 is required. However, ceramide-induced apoptosis failed to respond to z-VAD and different PARP cleavage was produced in ceramide-treated cells, suggesting that PARP cleavage between Asp214 and Gly215 by caspase 3 does not play a major role in ceramide-induced apoptosis, and a different apoptotic pathway is involved. Interestingly, a 105 kD band was found only in ceramide-treated cells, and appearance of this band was not affected by z-VAD. PARP was known to be cleaved by caspase 3 at the EVDG sequence between Asp214 and Gly215 amino acid residues, which results in an 85 kD product (Bose *et al.*, 1995). Another EVDG sequence was found at 73 site of PARP and cleavage at this site would result in a 105 kD fragment. Whether the 105 kD fragment is a result from cleavage at this potential site and what caspases-like activity is involved in the cleavage are interesting questions and deserve further investigation. Nevertheless, the results suggest the mechanism of UV and ceramide-induced apoptosis is apparently different.

Consistent with our conclusion, several recent observations also suggest that ceramide may not be involved in Fas- or TNF-induced apoptosis (Hus *et al.*, 1998; Karasavvas and Zaker, 1999; Sillence and Allan, 1997). However, the major evidence in these studies is that apoptosis induction was inconsistent with absolute ceramide levels. It should be noticed that the relative ceramide elevation in acute phase following stress stimuli, rather than absolute level of ceramide in later

(persistent) phase, was suggested to play a key role in stress-induced apoptosis (Hannun, 1996; Verheij *et al.*, 1996). Thus, these studies could not rule out the possibility that the apoptotic program was initiated by relative minor ceramide elevation in acute phase rather than absolute ceramide level in later (persistent) phase following stress. In the current study, we exclude this possibility by comparing the endogenous ceramide elevation by UV (Figure 2a) and sensitivity to exogenous ceramide in both parental cells and E1A transfectants (Figures 2b and 3). Furthermore, the additional kinetic (Figure 4) and biochemical (Figure 5) data provides unequivocal evidence to conclude that ceramide is unlikely to be the second messenger for UV-induced apoptosis.

Materials and methods

Reagents

C2-ceramide was obtained from Sigma (St. Louis, MO, USA). The interleukin-1β-converting enzyme (ICE) protease inhibitor z-VAD was obtained from Enzyme System Products (Livermore, CA, USA).

UV irradiation

Cells, grown to 50–80% confluence, were subjected to UV irradiation at room temperature with a source of UV-C light (254 nm). The dose of UV radiation was verified by UVX radiometer (UVP, Inc.).

Cell cultures

E1A transfectants of the murine melanoma cell line K1735 M2 and the human breast cancer cell line MDA-MB-231 were obtained by transfection with pE1A-neo which encodes both the E1A gene and neomycin-resistant gene (Deng *et al.*, 1998; Pierceall *et al.*, 1992; Shao *et al.*, 1997, 1998). Control plasmid transfectants (KSP) were obtained by transfection with backbone plasmid that encodes neomycin-resistant gene only (Deng *et al.*, 1998; Pierceall *et al.*, 1992; Shao *et al.*, 1997, 1998). The E1A mutants dl1101 and dl1108, which contain deletion mutations at the N-terminus or the CR2 domain, respectively, were obtained from Stanley Bayley (Westwick *et al.*, 1995). Mutant E1A transfectants (K01.9 for dl1101 and K08.5 for dl1108) were obtained by transfecting K1735 M2 cells with the corresponding mutant E1A plasmid plus pSV-neo, which encodes the neomycin resistance gene. Stable transfectants were selected in growth medium containing 500 μg/ml G418, and maintained in medium containing 200 μg/ml G418.

Immunoblot

Immunoblot analysis was performed as previously described (Deng *et al.*, 1998). The primary antibodies used for immunoblot were the monoclonal antibody M58 against the E1A proteins (Pharmingen, CA, USA), and the monoclonal antibody C2-10 (as Ab#1) against poly (ADP-ribose) polymerase (PARP) (Pharmingen, San Diego, CA, USA) (Kaufmann *et al.*, 1993) and the polyclonal antibody (as Ab#2) against cleaved PARP (New England Biolabs, Beverly, MA, USA). The blots were incubated with horseradish-conjugated rabbit antimouse immunoglobin (Bio-Rad Laboratories, Richmond, CA, USA) and enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, UK).

Flow cytometry, DNA fragmentation assays

Apoptotic cells were analysed by fluorescence-activated cell sorting (FACS) assay as described elsewhere (Deng *et al.*, 1998). Briefly, apoptotic cells as well as attached cells of each sample were harvested, washed once with phosphate buffered saline (PBS), and then fixed with 70% ethanol overnight at 4°C. Before analysis, cells were washed once with PBS, and then with fluorochrome solution (50 μg/ml propidium iodide (Sigma, St. Louis, MO, USA), in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma)). The percentage of subdiploid cells was considered to reflect that of apoptotic cells. Fragmented DNA was analysed by using a modified method described previously (Deng *et al.*, 1998; Nicoletti *et al.*, 1991).

Quantification of endogenous ceramide

Ceramide was quantified by the diacylglycerol kinase assay as described previously (Bose *et al.*, 1995; Dressler and Kolesnick, 1990). In brief, cells were pelleted by centrifugation and suspended in 150 μl of buffered saline solution (BSS), and then the suspended cells were extracted with 800 μl of chloroform:methanol:HCl (100:100:1 v/v/v). Lipid in the organic phase was dried in a speed vapor apparatus. Samples were dissolved in 0.1 M KOH methanol, incubated for 1 h at 37°C to remove glycerophospholipids, extracted with chloroform:methanol:1 N HCl (100:100:1) once and then dried. The dried samples were then dissolved in 60 μl of a reaction mixture containing *E. coli* diacylglyerol kinase and [γ-^{32}P]ATP, and incubated for 1 h at room temperature. The reaction was stopped by adding 100 μl of chloroform and 100 μl of methanol. The mixture was extracted once, then dried. [^{32}P]ceramide was dissolved in 5 μl thin layer chromatographic (TLC) plate using the solvent system chloroform:methanol:acetic acid (65:15:5). Incorporated γ-^{32}P was quantified by X-ray film and liquid scintillation counting (Bose *et al.*, 1995; Dressler and Kolesnick, 1990).

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Axl-Gas6 Interaction Counteracts E1A-Mediated Cell Growth Suppression and Proapoptotic Activity

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The adenovirus type 5 early region 1A gene (*E1A*) has previously been known as an immortalization oncogene because E1A is required for transforming oncogenes, such as *ras* and *E1B*, to transform cells in primary cultures. However, E1A has also been shown to downregulate the overexpression of the *Her-2/neu* oncogene, resulting in suppression of transformation and tumorigenesis induced by that oncogene. In addition, E1A is able to promote apoptosis induced by anticancer drugs, irradiation, and serum deprivation. Many tyrosine kinases, such as the epidermal growth factor receptor, Her-2/Neu, Src, and Axl, are known to play a role in oncogenic signals in transformed cells. To study the mechanism underlying the E1A-mediated tumor-suppressing function, we exploited a modified tyrosine kinase profile assay (D. Robinson, F. Lee, T. Pretlow, and H.-J. Kung, Proc. Natl. Acad. Sci. USA 93:5958–5962, 1996) to identify potential tyrosine kinases regulated by E1A. Reverse transcription (RT)-PCR products were synthesized with two degenerate primers derived from the conserved motifs of various tyrosine kinases. A tyrosine kinase downregulated by E1A was identified by analyzing the *Alu*I-digested RT-PCR products. We isolated the DNA fragment of interest and found that E1A negatively regulated the expression of the transforming receptor tyrosine kinase Axl at the transcriptional level. To study whether downregulation of the Axl receptor is involved in E1A-mediated growth suppression, we transfected *axl* cDNA into E1A-expressing cells (ip1-E1A) to establish cells that overexpressed Axl. The Axl ligand Gas6 triggered a greater mitogenic effect in these ip1-E1A-Axl cells than in ip1-E1A control cells and protected the Axl-expressing cells from serum deprivation-induced apoptosis. These results indicate that downregulation of the Axl receptor by E1A is involved in E1A-mediated growth suppression and E1A-induced apoptosis and thereby contributes to E1A's antitumor activities.

The adenovirus type 5 early region 1A gene (*E1A*), the first viral gene expressed in a cell after adenovirus infection, encodes two major proteins, 243R (12S) and 289R (13S), that are produced by alternative splicing (40). The primary function of these two proteins is to activate viral promoters of early genes, including *E1B*, *E2A*, *E3*, and *E4*, during a permissive viral infection by modifying the host cell transcriptional apparatus, thereby resulting in host cell immortalization or transformation (2). Cellular genes that are transcriptionally activated by the E1A proteins include those encoding β-tubulin (42), heat shock proteins (22), c-Fos (10, 37), c-Jun (10), JunB (10), and c-Myc (37). E1A can also repress several viral and cellular genes at the transcriptional level, such as the simian virus 40 enhancer (3, 50), the polyomavirus enhancer (3, 49), the immunoglobulin heavy-chain gene (21), the cytochrome P450 gene (41), the insulin gene (43), and the *Her-2/neu* gene (57).

Adenovirus type 5 *E1A* is known to be an immortalization oncogene because of the fact that some transforming oncogenes, such as *ras* and *E1B*, although able to induce transformation of immortalized rodent cell lines, require E1A to transform cells in primary cultures. However, adenovirus type 5 E1A alone cannot transform established cell lines (5, 23, 36). Furthermore, there exist many established cell lines that are immortalized but nontumorigenic. As a matter of fact, we have previously shown that E1A could be a tumor suppressor for *Her-2/neu*-transformed cells by transcriptional repression of the oncogene (52, 57). In addition to downregulating Her-2/Neu expression, E1A has multiple antitumor effects on tumor cells that do not overexpress Her-2/Neu, including reversion of transformation (12, 15, 16, 28, 55), inhibition of metastasis (17, 28, 53, 54, 56), and induction of apoptosis (9, 11, 33, 45). To understand the detailed mechanisms underlying E1A's antitumor activities, we examined the E1A-regulated tyrosine kinases and identified Axl, whose expression was suppressed by E1A.

The Axl tyrosine kinase (p140) is the prototype of a family of transmembrane receptors called UFO that include Sky and Eyk. These receptors have a unique extracellular composition of immunoglobin-like and fibronectin type III-like domains (13, 30, 47). Similar domain architectures have been found in adhesion molecules of the cadherin and immunoglobulin superfamily and in receptor tyrosine phosphatases such as PTPμ and PTPκ, suggesting that Axl family members may mediate cell adhesion and intracellular signaling. Increased expression of the Axl receptor can transform NIH 3T3 cells and renders the transformed cells highly tumorigenic in nude mice (30).

All members of the UFO family can transform NIH 3T3 cells. When transfected, the *axl* gene transforms NIH 3T3 cells by overexpression (30). Since no genetic rearrangements or mutations have been found, its transforming ability is most probably a consequence of normal receptor overexpression. The Axl ligand Gas6 is a vitamin K-dependent growth-poten-

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tiating factor (26, 44, 48). The ligand contains a γ-carboxyglutamic acid-rich domain and four epidermal growth factor-like repeats. It is widely secreted by most tissues, particularly those of the lung, intestine, and vascular endothelium (26). Under serum-starved conditions, Gas6 has a mitogenic effect on growth-arrested cells (1, 19, 20). Gas6-Axl signaling also seems to play a critical role in modulating cellular responses to adhesion; in one study, Gas6 stimulated the binding of Axl-expressing monoblast U937 cells to phosphatidylserine, a phospholipid marker used by macrophages to identify dying cells (29). Gas6 also functions as a novel chemoattractant that induces Axl-mediated migration of vascular smooth-muscle cells, suggesting that the Gas6-Axl interaction may enhance cell migration in conditions involving vascular damage (14). In addition, the Axl receptor has been found to be highly expressed in metastatic colon carcinoma (8), melanoma (32), and sarcoma (51) cells, implying that this receptor is involved in cancer invasion and metastasis.

The published data, taken together, suggest that E1A is associated with a tumor suppressor function in human tumor cells and that the Axl receptor behaves as an oncoprotein when it is overexpressed. To study the mechanism underlying E1A's tumor-suppressing activities, we focused on E1A-regulated tyrosine kinases. In the current study, we found that E1A can downregulate the expression of the Axl receptor and that the Gas6-Axl interaction can counteract E1A-mediated growth inhibition and proapoptotic activity, suggesting that downregulation of Axl by E1A may contribute to E1A-mediated antitumor activities.

MATERIALS AND METHODS

Cell lines and cell cultures. SKOV3.ip1 (abbreviated ip1) is a subline of the SKOV3 ovarian cancer cell line. 2774 C-10 (abbreviated 2774) is a human ovarian cancer cell line. The E1A transfectants were designated ip1-E1A and 2774-E1A. The transfectants of the *E1A* frameshift mutant were designated ip1-efs and 2774-efs. Cells were grown in Dulbecco's modified Eagle's medium plus F12 medium (1:1; GIBCO-Bethesda Research Laboratories [BRL]) supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. ip1-efs, ip1-E1A, 2774-efs, and 2774-E1A cells were maintained in medium containing neomycin at 500 μg/ml (Boehringer Mannheim).

Tyrosine kinase display assay. Total RNA was isolated with the TRIzol reagent (GIBCO-BRL), and the tyrosine kinase display was carried out by a modified form of the method of Robinson et al. (34, 35). Reverse transcription (RT)-PCR was performed with degenerate primers derived from conserved motifs in the activation loop of the catalytic domains of various tyrosine kinases as follows: primer 1 (sense primer), 5′-AARRTTDCNGAYTTYGG encoding the amino acid sequence K[V/I][S/C/G]DFG; primer 2 (antisense primer), 5′-RHAIGMCCAIACRTC encoding the amino acid sequence DVW[S/A][F/Y]. The mixed bases were defined as follows: N = A + C + T + G, D = A + T + G, H = A + T + C, R = A + G, Y = C + T, M = A + C, and I = deoxyinosine. Single-stranded cDNA was synthesized with a kit provided by GIBCO-BRL. Primer 1 was labeled with [γ-^{32}P]ATP (Amersham Life Science) and T4 polynucleotide kinase (New England Biolabs) before PCR (*Taq* DNA polymerase; Fisher Biotech). The RT-PCR products were analyzed by gel electrophoresis with an 8% polyacrylamide gel. DNA was stained with ethidium bromide at 1 μg/ml. The ~150-bp bands were excised from the polyacrylamide gel. DNA was eluted, precipitated, and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Equal amounts of radioactive DNA (10^5 cpm) of each cell type were digested with various restriction enzymes recognizing four bases, resolved with a 6% DNA sequencing gel, and then subjected to autoradiography.

Cloning of the band of interest revealed by the tyrosine kinase display assay. In the tyrosine kinase display assay, we found that the E1A proteins downregulated a tyrosine kinase whose catalytic domain sequence possessed the *Alu*I site. This tyrosine kinase was tentatively named TK-Alu I. Because the TK-Alu I band shown in Fig. 1B had been digested by *Alu*I, we could not PCR amplify the TK-Alu I DNA by using the two primers described previously, because the annealing sequence of the TK-Alu I for primer 2 had been separated from the radioactive sequence shown in Fig. 1B by the *Alu*I digestion. To clone TK-Alu I, we established a tyrosine kinase cDNA library from the RT-PCR products of ip1 cells with the TA cloning strategy. At first, all of the RT-PCR products of the 150-bp DNA fragments were ligated into the TA cloning vector PCR2.1 (Invitrogen Inc.) to make a cDNA library of tyrosine kinases. The library was amplified in *Escherichia coli* DH10B. Then, we performed a large-scale preparation of the *Alu*I-digested RT-PCR product. The TK-Alu I band was excised, and the DNA was eluted from the sequencing gel. A TK-Alu I probe was made by synthesizing single-stranded DNA by PCR in a mixture containing primer 1, [α-^{32}P]dCTP, and the eluted TK-Alu I DNA. The radiolabeled probe was used to screen the tyrosine kinase cDNA library described above, the positive clones were picked out, and the plasmid DNA was purified and subjected to PCR as described for the tyrosine kinase display assay. RT-PCR and plasmid-PCR products were digested with *Alu*I and then analyzed by sequencing gel electrophoresis to determine whether the selected clones contained the TK-Alu I insert. Finally, the truly positive TK-Alu I clone was sequenced and that sequence was identified by comparison with the GenBank database sequences of the National Center for Biotechnology Information by using the BLAST algorithm.

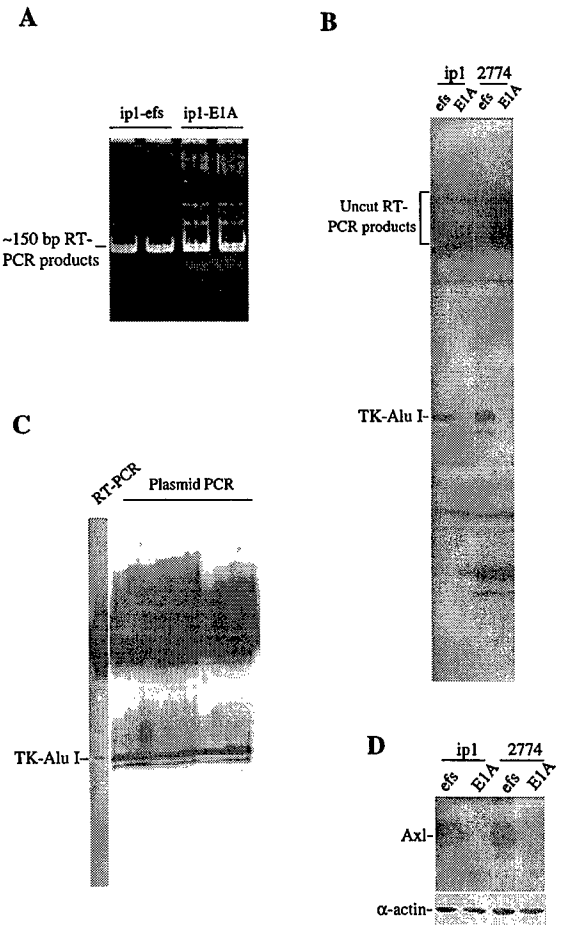

FIG. 1. (A) The 150-bp RT-PCR products representing the activation loop of the catalytic domains of various tyrosine kinases. RNA was isolated by using the TRIzol reagent. Single-stranded cDNA was synthesized with a GIBCO-BRL kit. Primer 1 was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase before PCR. The RT-PCR products were analyzed by 8% PAGE. DNA was stained with ethidium bromide at 1 μg/ml. SKOV3.ip1 is a subline of the SKOV3 ovarian cancer cell line; ip1-efs is ip1 cells transfected with an E1A frameshift mutant construct. (B) Tyrosine kinase display assay showing downregulation of a tyrosine kinase, designated TK-Alu I, in E1A-transfected cells. The RT-PCR products shown in panel A were excised and eluted from the gel. The eluted DNAs of equal radioactivity were digested with the restriction enzyme *Alu*I and then fractionated with a 6% DNA sequencing gel. 2774 is the ovarian cancer cell line 2774-C10. (C) Tyrosine kinase display assay of bacterial clones obtained from the tyrosine kinase cDNA library screening. The TK-Alu I probe was PCR labeled and used to screen a cDNA library of tyrosine kinases. The positive clones were picked out, and the plasmid DNA was purified and subjected to PCR as described for panel A. The products of both RT-PCR from ip1-efs cells and plasmid PCR from bacterial clones were digested with *Alu*I and then resolved with a 6% DNA sequencing gel. The TK-Alu I band was seen in all of the plasmid PCR clones. (D) Immunoblotting analysis showing decreased level of the Axl receptor protein in E1A-expressing ip1 and 2774 cells. Western blot analysis was performed as described in Materials and Methods, and the Axl receptor was detected by treating the transblot with an anti-Axl antibody. As a gel loading control, the same blot was reprobed with an anti-α-actin antibody.

Nuclear run-on assay. The nuclear run-on assay was performed as described previously (27). Briefly, cells were washed with phosphate-buffered saline (PBS) and lysed in ice-cold buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The nuclei were then pelleted by centrifugation and suspended in buffer A (50 mM Tris [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol). The nascent RNA chains were elongated by mixing the nuclear suspension with an equal volume of the reaction buffer (10 mM Tris [pH 8.0]; 5 mM $MgCl_2$; 0.3 M KCl; 5 mM dithiothreitol; 1 mM each ATP, CTP, and GTP; 0.1 mCi [α-^{32}P]UTP). After incubation at 30°C for 30 min, the ^{32}P-labeled RNA was purified with TRIzol (GIBCO-BRL). Samples (5 μg) of plasmids, pcDNA3, pcDNA3-Axl, and pcDNA3–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were denatured and immobilized on Hybond N membranes. The membranes were prehybridized in a solution of 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA), 2× Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), and salmon sperm DNA at 100 μg/ml for 2 h at 42°C. Equal amounts of radioactivity (1.0×10^6 cpm) from the test samples were hybridized with the immobilized DNA at 42°C for 24 h. The membranes were then washed for 60 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 55°C and then for 30 min with 0.2× SSC–0.1% SDS at 55°C and finally exposed to X-ray film at −70°C.

Establishment of stable cell lines. The *axl* cDNA was cloned into a pCEP4 vector carrying the hygromycin phosphotransferase gene (*hph*). Expression of the *axl* and *hph* genes was driven by the cytomegalovirus promoter and the thymidine kinase promoter, respectively. The *axl* gene transfection was carried out by using DOTAP liposomes (24). Briefly, 6 μg of plasmid pCEP4-Axl along with the DOTAP liposome mixture was transfected to 1.0×10^6 ip-E1A cells cultured in a 3-cm-diameter tissue culture dish. Approximately 4 h after transfection, the cells were washed with PBS, cultured in fresh medium for 24 h, and then split 1:20. The cells were then grown in a selection medium containing hygromycin B (Boehringer Mannheim) at 20 μg/ml and neomycin at 500 μg/ml for 3 to 5 weeks, after which individual hygromycin-resistant clones were picked out and expanded to mass culture.

Immunoblotting. Immunoblotting analyses were performed as described elsewhere (46). Briefly, cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes. The membranes were treated separately with an anti-Axl (Santa Cruz Biotech), an anti-E1A protein (Oncogene Research), or an anti-α-actin (Amersham Life Science) antibody and then incubated with peroxidase-conjugated secondary antibodies and detected by the enhanced-chemiluminescence method (Amersham Life Science). For the analysis of Gas6 expression, supernatants of cultures were subjected first to SDS-PAGE and then to immunoblotting with an anti-Gas6 polyclonal antibody (Santa Cruz Biotech).

[^{3}H]thymidine incorporation assay. Cells were trypsinized, washed twice with PBS, and then seeded onto 96-well plates at 2,000/well. To the wells was added 10 μCi of [^{3}H]thymidine (Amersham Life Science) at specific times. The cells were then incubated at 37°C for 8 h and then processed by a cell harvester (Cambridge Technology Inc.). Radioactivity was determined with a liquid scintillation counter.

Analysis of [^{3}H]thymidine incorporation after the *gas6* cDNA transfection. The medium used to do the *gas6* cDNA transfection and other studies related to Gas6 contained 4 μM menadione sodium bisulfite, a vitamin K analog (Sigma). The *gas6* cDNA, whose expression was driven by the cytomegalovirus promoter, was cloned in a pcDNA3 vector. Cells (1.0×10^6) were plated in a 3-cm-diameter tissue culture dish and cultured in drug-free medium for 24 h, and then 4 μg of the vector pcDNA3 or plasmid pcDNA3-Gas6 was transfected into ip1-efs, ip1-E1A-pCEP4, and three independent clones of ip1-E1A-Axl cells by using DOTAP liposomes. Cells were exposed to the DNA-liposome composites for 8 h, washed twice with PBS, and cultured in serum-supplemented medium for 24 h. Cell were then trypsinized, washed twice with PBS, and then seeded onto 96-well plates at 2,000/well. At 24, 48, and 72 h thereafter, cells were subjected to [^{3}H]thymidine incorporation. Alternatively, 24 h after being seeded onto 96-well plates, cells were serum deprived for another 24 h and then subjected to [^{3}H]thymidine incorporation.

Analysis of the phosphorylation state of the Axl receptor. Cells for this assay were lysed in a buffer containing 20 mM Tris [pH 7.2], 150 mM NaCl, 50 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 25 μg/ml, and aprotinin at 25 μg/ml. The lysates were cleared by centrifugation and immunoprecipitated with 5 μg of anti-Axl polyclonal antibody (Santa Cruz), followed by 20 μl of protein A-agarose (Boehringer Mannheim). The immunocomplexes were washed with lysis buffer, resolved by SDS–10% PAGE, transferred to a nitrocellulose membrane, and then detected with an antiphosphotyrosine antibody (Oncogene Research).

Analysis of apoptosis by flow cytometry. For analysis of apoptosis by flow cytometry, 1.0×10^6 cells were transfected with *gas6* cDNA by using the LPD1 liposome (24). At 24 h after transfection, cells were serum starved for 48 h, harvested by trypsinization, washed twice with PBS, and then fixed in 75% ethanol at 4°C overnight. The fixed cells were washed twice with PBS and suspended in 1 ml of PBS containing 0.5% Tween 20, to which were added 10 μg of RNase and 10 μg of propidium iodide. The propidium iodide-stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson). Apoptotic cells were determined by the percentage of cells in the sub-G_1 region.

RESULTS

Identification of the tyrosine kinase Axl that was downregulated by the E1A proteins. To identify the E1A-regulated tyrosine kinases, we compared the tyrosine kinase expression profiles of cancer cell lines with those of their E1A transfectants by using a novel tyrosine kinase display assay (34), a modification of the tyrosine kinase profile approach (35). Briefly, the entire collection of expressed tyrosine kinases was generated by RT-PCR using degenerate primers corresponding to the highly conserved DFG and DVW motifs. This would yield a relatively homogeneous band of approximately 150 bp for virtually all tyrosine kinases. Different kinases would then be differentiated by digestion with restriction enzymes, based on the characteristic sizes predicted from the GenBank data. To help visualize the restriction products, the 5′ end of the sense primer was radiolabeled with [γ-^{32}P]ATP and the digested products were resolved in a DNA sequencing gel and then autoradiographed. An example of the polyacrylamide gel-resolved and ethidium bromide-stained total RT-PCR products in the range of 150 bp is shown in Fig. 1A. Restriction enzyme digestion of the RT-PCR products would reduce the lengths of certain radiolabeled DNA fragments, which would migrate faster than uncut fragments in a DNA sequencing gel. A tyrosine kinase cDNA fragment, tentatively named TK-AluI, that had been digested with *Alu*I displayed a differential expression pattern in E1A-transfected cells compared with the parental controls (Fig. 1B). This band exhibits a very low radiointensity in both the E1A-expressing ip1 and 2774 ovarian cancer cells compared with the control cells. To confirm that the lower expression of TK-Alu I was due to the expression of E1A, we subjected the breast cancer cell line MDA-MB231 and its E1A stable transfectant (231-E1A) to the same analysis and found downregulation of Axl in 231-E1A cells (data not shown). Thus, it is likely that the mRNA level of the TK-Alu I gene was negatively regulated by E1A. The size of the *Alu*I-digested band predicts that it may be the tyrosine kinase Axl.

To confirm that the TK-Alu I gene encodes Axl but not any fortuitous RT-PCR product, we set out to clone this product. A radioactive probe was prepared by PCR using the TK-Alu I DNA as a template, and that probe was used to screen a tyrosine kinase cDNA library established by ligating the RT-PCR products synthesized from ip1 cells to the TA cloning vector PCR2.1. As expected, the positive clones showed the same TK-Alu I band upon tyrosine kinase display (Fig. 1C). DNA sequencing revealed that TK-Alu I indeed represents the Axl kinase. Once we identified the product of the TK-Alu I gene as Axl, we analyzed Axl protein levels in cancer cell lines and their E1A transfectants. We found that expression of the Axl protein was repressed in E1A transfectants (Fig. 1D). Thus, both protein and mRNA expression findings support the inhibition of Axl expression by E1A.

Repression of *axl* gene expression by E1A at the transcriptional level. The foregoing results suggest that E1A can negatively regulate the expression of both the *axl* transcripts and the Axl protein. We next used a nuclear run-on assay to determine whether E1A represses transcription of the *axl* gene. The radiolabeled transcripts derived from the nuclei of ip1-efs cells hybridized strongly with the *axl* cDNA, but the radiolabeled transcripts derived from ip1-E1A cells gave no hybridization signal (Fig. 2). Hybridization of the same radiolabeled transcripts to GAPDH cDNA, a housekeeping gene, produced equal signals in the ip1-efs and ip1-E1A cells. These results indicate that ip1-E1A cells produced little or no Axl mRNA. To determine whether this phenomenon was caused by transcriptional repression of the *axl* gene or by posttranscriptional

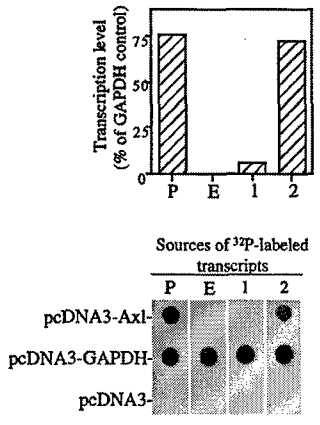

FIG. 2. Nuclear run-on assay showing transcriptional repression of the *axl* gene in ip1-E1A cells. ^{32}P-labeled transcripts were prepared from isolated nuclei by in vitro transcription with [α-^{32}P]UTP. Equal amounts of labeled RNA (10^6 cpm) were hybridized to Hybond N membranes containing the immobilized plasmids pcDNA3-Axl, pcDNA3-GAPDH, and pcDNA3 at 5 μg per blot. P, ip1-efs cells; E, ip1-E1A cells. Lanes: 1, membrane strip hybridized with the in vitro transcription product derived from an equal amount mixture of the nuclear suspensions of ip1-efs and ip1-E1A cells; 2, membrane strip hybridized with the in vitro transcription product derived from the nuclear suspension of ip1-efs cells, to which was added an equal amount of nuclear suspension of ip1-E1A cells after mRNA elongation was completed.

degradation of *axl* mRNA in ip1-E1A cells, we elongated mRNA from ip1-efs cells and then added a nuclear suspension of ip1-E1A cells to the products of in vitro transcription. The hybridization result (Fig. 2, lane 2) was the same as that seen in the lane representing ip1-efs. Thus, the nuclear suspension of ip1-E1A cells did not contain factors that accelerated degradation of the *axl* mRNA. In vitro transcriptional repression was also observed in a mixture of the nuclear suspensions of ip1 cells and ip1-E1A cells (Fig. 2, lane 1), suggesting that some factor in ip1-E1A cells is able to act in *trans* to suppress *axl* transcription in ip1-efs cells. Thus, we conclude that the rate of *axl* gene transcription was greatly inhibited in ip1-E1A cells compared with that in the control ip1-efs cells.

Establishment of Axl-overexpressing ip1-E1A cells. ip1-E1A cells grow more slowly than do the parental ip1 cells (58). To study whether downregulation of the Axl receptor by E1A might be involved in the E1A-mediated reduction of the growth rate of ip1-E1A cells, we established an ip1-E1A cell line that overexpresses Axl (ip1-E1A-Axl). The *axl* cDNA was cloned into the expression vector pCEP4 that carries the hygromycin phosphotransferase gene. The stable cell lines were established by transfection of the pCEP4-Axl plasmid into ip1-E1A cells and selection with hygromycin B. These ip1-E1A-Axl clones overexpressed the Axl receptor; the expression level in clone 2 was comparable to that of the ip1-efs cells (Fig. 3). To verify that E1A was still present in the stable clones, the same blot was reprobed with an anti-E1A antibody. Expression of α-actin was used as a gel loading control.

Promotion of mitogenesis by the Gas6-Axl interaction in ip1-E1A-Axl cells. To determine whether ip1-E1A-Axl cells might recover after growth rate reduction by E1A, we measured mitogenesis by determining [^{3}H]thymidine incorporation. Re-expression of the Axl receptor in ip1-E1A cells (ip1-E1A-Axl) had no significant effect on mitogenesis, compared to ip1-E1A-pCEP4 control cells that were transfected with the vector plasmid (Fig. 4A). Since previous reports had shown that activation of Axl is Gas6 dependent (1, 19, 20), we questioned whether the Axl receptor expressed in ip1-E1A-Axl cells requires the ligand Gas6 for activation.

To determine whether Gas6 could promote mitogenesis in ip1-E1A-Axl clones, cells were transfected with either vector pcDNA3 or plasmid pcDNA3-Gas6 and [^{3}H]thymidine incorporation was performed in the presence of serum at specific times thereafter. Gas6 produced a dose-dependent activation of the Axl receptor in ip1-E1A-Axl cells (Fig. 4B) with transfection of 4 μg of plasmid pcDNA-Gas6 to 10^6 ip1-E1A-Axl-2 cells giving the maximum phosphorylation of the Axl receptor and a plateau incorporation of [^{3}H]thymidine. Gas6 expression in culture supernatants was confirmed by Western blotting. These results indicate that the transfection assay we established can produce enough secreted Gas6 to induce Axl-dependent mitogenesis.

Using the above-described assay, we found that transiently expressed Gas6 had an increased mitogenic effect on ip1-E1A-Axl clones in the absence of serum compared with the same clones without Gas6 stimulation (Fig. 4C). Expression of Gas6 is shown in Fig. 4D-1. This protein had a lesser impact on mitogenesis in ip1-efs cells (1.6-fold increase) than on that in ip1-E1A-Axl cells (10-fold increase). For ip1-E1A-Axl-2 cells, Gas6 induced a 10-fold increase in mitogenesis in the absence of serum (Fig. 4C) but only a 3-fold increase in the presence of serum (4-μg lane in Fig. 4B), suggesting that serum contains other stimulating factors that cover the effect of Gas6.

To confirm the effect of the Gas6-Axl interaction, we analyzed the intrinsic phosphorylation of the Axl receptor in the presence or absence of Gas6 stimulation. Cells were transfected with either the pcDNA3-Gas6 plasmid or the control vector, serum deprived for 24 h, immunoprecipitated with an anti-Axl antibody, and Western blotted with an antiphosphotyrosine antibody. The increased tyrosine phosphorylation of the Axl receptor in the Gas6-stimulated ip1-E1A-Axl cells (Fig. 4D-2) correlates well with the enhanced mitogenic effect in ip1-E1A-Axl cells (Fig. 4C). The same blot was reprobed with an anti-Axl antibody as a control.

Protection of E1A-transfected cells from serum deprivation-induced apoptosis by the Gas6-Axl interaction. E1A-transfected cells are more sensitive to serum deprivation-induced apoptosis than are parental cells (11, 33). We found that Gas6 can induce mitogenesis in ip1-E1A-Axl cells in the absence of serum (Fig. 4C). To examine whether the Gas6-Axl interaction could also protect ip1-E1A-Axl cells from apoptosis triggered by serum deprivation, we transfected cells with plasmid pcDNA3-Gas6, deprived those cells of serum for 48 h, and then processed them for fluorescence-activated cell sorter

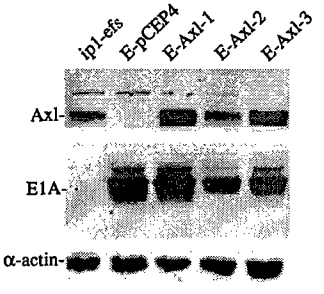

FIG. 3. Immunoblot showing re-expression of the Axl receptor in *axl*-transfected ip1-E1A cells. *axl* cDNA was cloned into the pCEP4 vector, and the DNA was transfected as described in Materials and Methods. Three clones of ip1-E1A-Axl (E-Axl-1, E-Axl-2, and E-Axl-3) showed increased expression of Axl compared to that of ip1-E1A-pCEP4 (E-pCEP4) control cells that were transfected with the pCEP4 vector. An equal amount of cell lysate for each cell type was resolved by SDS–10% PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane and then probed with anti-Axl, anti-E1A, and anti-α-actin antibodies.

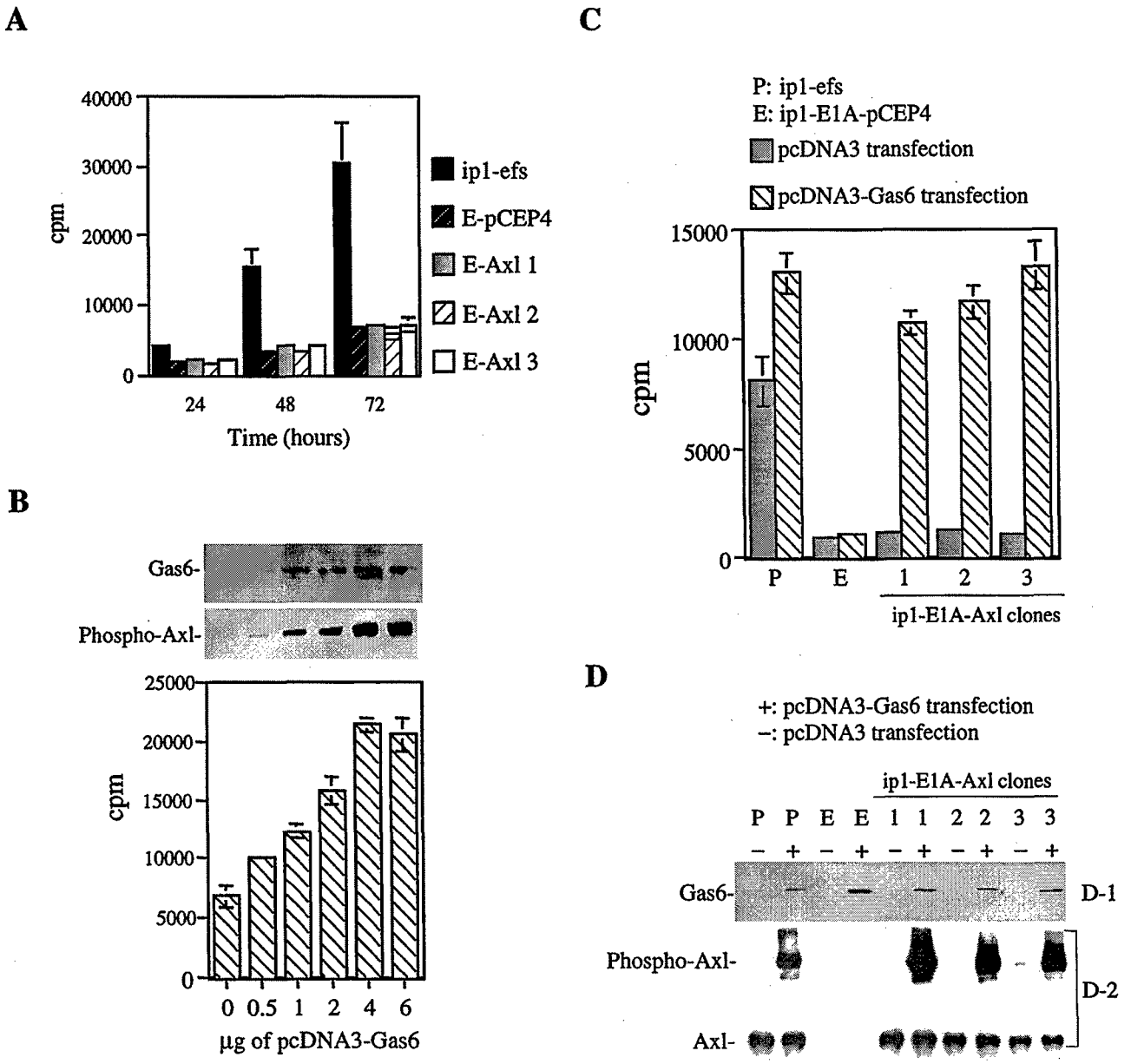

FIG. 4. Mitogenesis in ip1-efs, ip1-E1A, and ip1-E1A-Axl cells. (A) [^{3}H]thymidine incorporation showing no significant difference in mitogenesis between ip1-E1A-pCEP4 (E-pCEP4) cells and ip1-E1A-Axl clones (E-Axl-1, -2, and -3). Cells were seeded onto 96-well plates, [^{3}H]thymidine was added at 24, 48, and 72 h, and cells were incubated at 37°C for 8 h and then processed by a cell harvester. Each bar is the average of four replicates. (B) Dose-dependent activation of the Axl receptor in ip1-E1A-Axl-2 cells by *gas6* cDNA transfection. Various concentrations of pcDNA3-Gas6 plasmid DNA were transfected into 10^6 ip1-E1A-Axl-2 cells as described in Materials and Methods; 4 μg of plasmid pcDNA3-Gas6 was sufficient to cause maximum phosphorylation of the Axl receptor and plateau incorporation of [^{3}H]thymidine. Phosphorylation of the Axl receptor was analyzed by immunoprecipitation with an anti-Axl antibody, followed by Western blotting with an antiphosphotyrosine antibody. Gas6 expression was determined by immunoblotting of culture supernatants. All of the assays were performed 72 h after transfection and under serum-supplemented conditions. (C) The effect of Gas6 on serum-deprived ip1-E1A-Axl cells. Cells (10^6) were transfected with 4 μg of pcDNA3-Gas6 or vector pcDNA3 as described for panel B and seeded onto a 96-well plate at 2,000 cells/well 24 h after transfection. After 24 h of culture in serum-supplemented medium to allow the cells to attach to the wells, the cells were serum deprived for another 24 h and then subjected to [^{3}H]thymidine incorporation. Each bar is the average of triplicate samples. (D-1) Immunoblot showing Gas6 expression in pcDNA3-Gas6-transfected cells. Western blot analysis was performed with supernatants of cell cultures as described for panel B. (D-2) Increased phosphorylation of the Axl receptor in Axl-expressing cells transfected with *gas6* cDNA. Cells were transfected with pcDNA3-Gas6 or vector plasmid pcDNA3 as described for panel C. At 24 h after transfection, cells were serum deprived for 24 h and then lysed, immunoprecipitated with anti-Axl antibody, and subjected to Western blotting with an antiphosphotyrosine antibody. As a control, the same blot was reprobed with an anti-Axl antibody.

(FACS) analysis. Fewer Gas6-stimulated ip1-E1A-Axl cells than unstimulated cells were in the sub-G_1 region (Fig. 5A and B). Gas6 expression is shown in the lower part of Fig. 5B. The ip1-E1A-pCEP4 cells were sensitive to serum deprivation despite the presence of Gas6; the ip1-efs cells were less sensitive to serum withdrawal under the same conditions. These results indicate that Gas6 can protect E1A transfectants from serum deprivation-induced apoptosis if Axl is re-expressed.

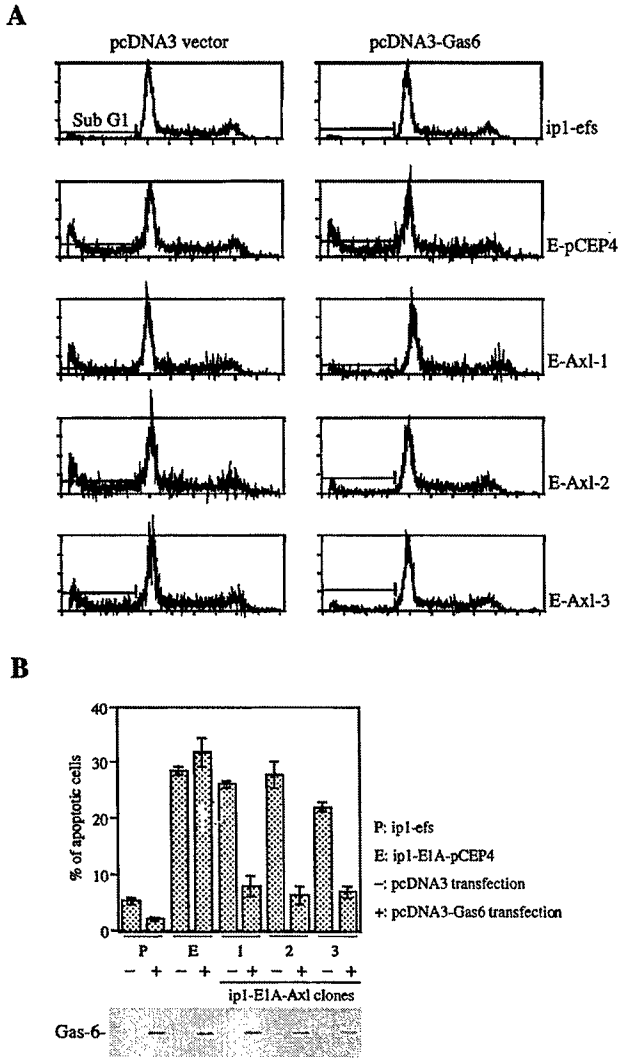

FIG. 5. Protection of Axl-expressing cells from serum deprivation-induced apoptosis by Gas6. (A and B) Decreased apoptosis in ip1-E1A-Axl cells by transiently expressed Gas6. Cells were transfected with *gas6* cDNA or control plasmid pcDNA3 as described for Fig. 4C. At 24 h after transfection, cells were serum deprived for 48 h and then subjected to FACS analysis. The apoptotic cells were determined by measuring the percentage of cells in the sub-G_1 region (A). Each bar in panel B is the average of triplicate samples. Expression of Gas6 in cells transfected with *gas6* cDNA is shown at the bottom of panel B. Supernatants from cells used for FACS analysis were subjected to SDS–10% PAGE. Western blot analysis was performed with an anti-Gas6 polyclonal antibody. Abbreviations for cell types are the same as those in Fig. 3 and 4C and D.

DISCUSSION

E1A has been shown to be associated with multiple antitumor activities, including transcriptional repression of the *Her-2/neu* gene (52, 57), suppression of transformation (12, 15, 16, 28, 55), inhibition of metastasis (17, 28, 53–55), and induction of apoptosis (9, 11, 33, 45). To further understand E1A-mediated tumor suppression, we focused on the tyrosine kinases that are regulated by E1A because tyrosine kinases usually play a pivotal role in the signal pathways that cause cellular transformation. In the current study, we used the tyrosine kinase display and nuclear run-on assays to confirm that the expression of the transforming receptor tyrosine kinase Axl is transcriptionally suppressed by E1A. Our experimental results indicate that the Gas6-Axl interaction counteracts E1A-mediated cell growth suppression and proapoptotic activity.

Multiple molecular mechanisms may account for the tumor- and metastasis-suppressing functions of E1A in different cancer cell types. The tumor-suppressing function has been explained, at least in part, as resulting from the induction of apoptosis through p53-dependent or p53-independent mechanisms (9, 45). E1A may also suppress tumor growth by modulating the response of tumor cells to immune cells, since this protein can sensitize transfected cells to the cytotoxic effects of tumor necrosis factor (TNF) (6, 38) and make target cells susceptible to NK cells and activated macrophages (7). In addition, E1A can abrogate NF-κB activation, resulting in susceptibility of cells to apoptotic stimuli such as TNF and γ irradiation (38, 39). As for the metastasis-suppressing function of E1A, one known mechanism is the transcriptional repression of various proteases that are important for tumor invasion and metastasis, including type IV collagenase (17, 18), interstitial collagenase, urokinase (17), and stromelysin (25, 31). In summary, E1A suppresses tumor growth and metastasis through cumulative changes in cellular gene expression, and many unknown mechanisms remain to be discovered.

Gas6 induced serum-starved NIH 3T3 cells to enter the cell cycle (19, 20); the signaling was shown to be transmitted through the stimulation of Axl tyrosine kinase with subsequent activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase (19, 20). Adding Gas6 to serum-starved NIH 3T3 cells also prevented cell death induced by complete serum removal and TNF addition, indicating that Gas6 acts as a survival factor for growth-arrested cells (1, 19, 20). In a study of fibroblasts from *axl* knockout mice, the absence of the Axl receptor resulted in higher levels of serum deprivation-induced apoptosis that could not be rescued by the addition of Gas6 (1). Instead of studying normal fibroblasts, we established an ovarian cancer model in which parental ip1 cells, unlike NIH 3T3 cells, were not sensitive to serum deprivation. In this model, downregulation of the Axl receptor by E1A rendered the E1A transfectant ip1-E1A susceptible to serum deprivation-induced apoptosis that was prevented by the Gas6-Axl interaction. In short, abrogation of Gas6-Axl signaling by E1A is involved in E1A-mediated suppression of cell growth and susceptibility to serum deprivation.

The mildly increased rate of [^{3}H]thymidine incorporation in Gas6-stimulated ip1-efs cells (Fig. 4C) is consistent with previous reports that Gas6 is a weaker mitogen than basic fibroblast growth factor and serum (1, 19). In the mitogenesis and apoptosis studies (Fig. 4C and 5), we found that ip1-efs cells were less responsive to Gas6 stimulation than were ip1-E1A-Axl cells, although both expressed Axl. Conversely, the mitogenic effect and resistance to serum withdrawal-induced apoptosis in ip1-E1A-Axl cells were dependent on Gas6 stimulation (Fig. 4C and 5), implicating the role of downregulation of Axl in E1A-mediated tumor suppression and E1A's other activities that render ip1-E1A-Axl cells more dependent than ip1-efs cells on Gas6 during serum depletion. One of these E1A activities may be downregulation of the receptor tyrosine kinase Her-2/Neu (52, 55, 57). Re-expression of Axl did not change the repressed expression of Her-2/Neu in ip1-E1A cells (data not shown). Overexpression of Her-2/Neu in ip1-efs cells (58) may be the reason why ip1-efs cells were less dependent on Gas6 stimulation (Fig. 4C) and more resistant to serum withdrawal-induced apoptosis (Fig. 5) than were ip1-E1A-Axl cells.

Expression of the activated *axl* gene in NIH 3T3 (AF6295) cells can result in cellular transformation (30). However, re-expression of the Axl receptor in ip1-E1A cells (ip1-E1A-Axl)

had no significant effect on mitogenesis as analyzed by [^{3}H]thymidine incorporation, compared to ip1-E1A-pCEP4 control cells that were transfected with the vector plasmid (Fig. 4A). This might be because the level of the Axl receptor in ip1-E1A-Axl clones was not as high as that in the AF6295 cells described by O'Bryan et al. (30). The expression of Axl in AF6295 cells is at least 10-fold more abundant than that in ip1-efs cells (data not shown). The mitogenesis study indicates that the receptor expressed in ip1-E1A-Axl cells requires the Axl ligand Gas6 for activation (Fig. 4B and D-2). Although Gas6 was able to stimulate mitogenesis in ip1-E1A-Axl cells, endogenous Gas6 expression could not be detected in the supernatants of parental ip1 cells or ip1-E1A cells (Fig. 4D-1 and 5B). This finding suggests that repression of the *axl* gene by E1A is not the main mechanism by which growth is suppressed in cultured ip1 cells. However, Gas6 secretion is ubiquitous in the human body (26), so Axl-mediated survival and mitogenic effects may be more important in whole organisms than in cultured cells. It should be mentioned that Axl is overexpressed in approximately 25% of primary breast cancers (4). Given that a tumor cell line can secrete detectable amounts of Gas6 protein, Gas6-Axl signaling may play a supportive role in preventing apoptosis induced by serum deprivation or TNF. On the other hand, Gas6 also can act as a chemoattractant and is involved in cell migration (14); several metastatic cell types have increased expression of the Axl receptor (7, 32, 50). Thus, suppression of the Axl receptor by E1A may partly explain E1A's metastasis-suppressing effect.

In summary, the known characteristics of the Axl receptor and E1A are consistent with our previous findings that E1A functions as a tumor suppressor. Further investigations are required to determine whether negative regulation of Axl by E1A is also involved in the decreased tumorigenicity and decreased metastatic potential observed in ip1-E1A cells. However, the present findings identify a second transforming receptor tyrosine kinase, Axl, that is repressed by E1A.

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Advances in Brief

Adenovirus 5 Early Region 1A Does Not Induce Expression of the Ewing Sarcoma Fusion Product EWS-FLI1 in Breast and Ovarian Cancer Cell Lines[1]

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Abstract

The adenovirus 5 early region 1A (E1A) can function as a tumor suppressor gene and is being used in clinical trials as a therapeutic agent for advanced breast, ovarian, and head and neck cancer. Recently, there has been a dispute regarding whether transfection with the *E1A* gene can induce expression of the Ewing sarcoma oncogenic fusion transcript EWS-FLI1 (Sanchez-Prieto *et al.*, Nat. Med., *5:* 1076–1079, 1999; Melot and Delattre, Nat. Med., *5:* 1331, 1999; Kovar *et al.*, Cancer Res., *60:* 1557–1560, 2000). In an effort to settle the controversy, we tested several stable E1A transfectants of cell lines MDA-MB-231, MCF-7, MDA-MB-435 (breast cancer), SKOV3-ip1 (ovarian cancer), and PC-3 (prostate cancer), as well as parental and vector-transfected controls, HEK 293 cells, and RD-ES (Ewing sarcoma) cells, for the EWS-FLI1 fusion product. The EWS-FLI1 transcript could not be identified with reverse transcription-PCR in any of the 13 E1A-transfected cell lines analyzed. Furthermore, the EWS-FLI1 fusion protein could not be detected by Western blot analysis in E1A-transfected cell lines. These results suggest that E1A transfection does not necessarily lead to expression of the oncogenic EWS-FLI1 fusion transcript. Thus, the potential induction of this gene rearrangement by E1A gene therapy is unlikely to be clinically significant in the treatment of advanced malignant disease.

Introduction

The adenovirus E1A[3] can function as a tumor suppressor gene (reviewed in Refs. 1 and 2). E1A can down-regulate the *erb*B2 oncogene to suppress tumorigenesis, and it can suppress metastasis by additional molecular mechanisms. Furthermore, E1A sensitizes cells to cellular host immune responses and to apoptotic agents such as anticancer drugs, tumor necrosis factor, and irradiation. The E1A protein is under active investigation as a potential therapeutic agent. A Phase I clinical trial of *E1A* gene therapy for patients with advanced breast and ovarian cancer has recently been completed (3), and Phase II trials are currently in progress.

Ewing sarcoma 11;22 translocation results in a rearrangement between EWS on chromosome 22q12 and FLI1 on chromosome 11q24, leading to a chimeric transcription factor, EWS-FLI1, that has potent transforming activity (4). Sanchez-Prieto *et al.* (5) have recently reported that the adenoviral E1A protein has the ability to induce expression of the EWS-FLI1 fusion protein. However, data from the studies of Melot and Delattre (6) and Kovar *et al.* (7, 8) do not support this concept. De Alava *et al.* (9) have suggested that this discrepancy may be due to the types of subclones and assay conditions used. Because of this controversy and because the induction of the oncogenic EWS-FLI1 transcript by E1A transfection could have significant consequences in the setting of *E1A* gene therapy, we tested stable, E1A-transfected breast and ovarian cancer cell lines for the expression of the EWS-FLI1 fusion product.

Materials and Methods

Cell Cultures, Plasmids, and Retroviral Vectors. The human breast cancer cell lines MCF-7 and MDA-MB-231 and ovarian cancer cell line SKOV3-ip1 with and without transfection of E1A were routinely maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). Stable cells expressing wild-type E1A or 12S E1A were established by transfecting cells with the E1A-expressing plasmid pE1A-neo or 12S E1A plasmid DNA plus pSV-neo. The transfectants were grown under the same conditions as controls, except that G418 was added to the culture medium. Expression of E1A was verified by Western blot analysis using monoclonal anti-E1A antibody M58 (Oncogene Science, Cambridge, MA). The human Ewing sarcoma cell line RD-ES was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 15% fetal bovine serum.

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[3] The abbreviations used are: E1A, 5 early region 1A; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

RT-PCR. Total RNA was isolated from cell lines with Trizol reagent (Life Technologies, Inc.). One μg of total RNA was reverse transcribed after priming with oligo(dT) primers using the Superscript Preamplification System (Life Technologies, Inc.). The single-stranded cDNA was amplified by 40 PCR cycles of denaturing at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 72°C for 30 s. RNA from the Ewing sarcoma cell line RD-ES was used as a positive control. Primers described previously by Sanchez-Prieto *et al.* (5) were used: (*a*) 11.3 FLI1, 5′-ACTCCCCGTTGTTGGTCCCCTCC-3′; (*b*) 22.3 EWS, 5′-TCCTACAGCCAAGCTCCAAGTC-3′; (*c*) 11.4 FLI1, 5′-CAGGTGATACAGCTGGCG-3′; and (*d*) 22.4 EWS, 5′-CCAACAGAGCAGCAGCTAC-3′. Nested PCR was first performed using the 11.3 FLI1 and 22.3 EWS primers and then performed using the 11.4 FLI1 and 22.4 EWS primers. Expression of the E1A transcripts was confirmed by RT-PCR using primers 5′-GCGCCTGCTATCCTGAGA-3′ and 5′-CCGCTCGAGTTATGGCCTGGGGCGTTTACA-3′. Amplification of GAPDH was performed as a control using primers 5′-AAGGTGAAGGTCGGAGTCAAC-3′ and 5′-CATGAGTCCTTCCACGATACC-3′. Amplified products were analyzed by 1.5% agarose gel electrophoresis.

Cloning and Sequencing. RT-PCR using the 11.4 FLI1 and 22.4 EWS primers (Fig. 1*a*) and the 11.3 FLI1 and 22.4 EWS primers was performed with RNA from the E1A-transfected MDA-MB-231 cell line and the RD-ES sarcoma cell line. The amplified products were subcloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA). The recombinant plasmids were sequenced and compared with GenBank database sequences from the National Center for Biotechnology Information using the BLAST algorithm.

Southern Blot Analysis. RT-PCR products were separated by 1.5% agarose gel electrophoresis and blotted onto nylon membranes (GeneScreen; New England Nuclear Life Science Products, Boston, MA). The membranes were hybridized in RapidHyb buffer (Amersham Corp, Arlington Heights, IL) and probed with the RD-ES 11.4 FLI1/22.4 EWS fusion fragment subcloned previously. The probe was randomly labeled with [^{32}P]dCTP using the random primers DNA labeling system (Life Technologies, Inc.). The 22.4 EWS oligonucleotide was radiolabeled with [γ-^{32}P]ATP with T4 polynucleotide kinase (New England Biolabs, Beverly, MA).

Western Blot Analysis. Western blot analysis of whole cell extracts was accomplished using standard procedures. Briefly, cells were collected and lysed with buffer A [20 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 0.15 M NaCl, 1 mM β-mercaptoethanol, 1 mM Na_3VO_4, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% (w/v) bromphenol blue]. Samples were heated at 100°C for 5 min and separated by 10% SDS-PAGE. After transfer, membranes were incubated with either the M73 antibody (Oncogene Sciences, Cambridge, MA) against E1A protein, the antibody against FLI1 (PharMingen, San Diego, CA), or the N-18 antibody against EWS (Santa Cruz Biotechnology, Santa Cruz, CA). As a loading control, membranes were also incubated with an antibody against actin (Boehringer Mannheim, Indianapolis, IN).

Results

E1A-transfected Cell Lines Do Not Express the EWS-FLI1 Fusion Transcript. Stable E1A transfectant of breast cancer cell line MDA-MB-231 (231-E1A), parental and vector-transfected controls, E1A-expressing HEK 293 cells, and RD-ES (Ewing sarcoma) cell lines were tested for the EWS-FLI1 fusion transcript. When RT-PCR was performed using the primer set 11.4 FLI1 and 22.4 EWS (see "Materials and Methods"), two products (130 and 250 bp) were detected in the 231-EIA and HEK 293 cells but not in the parental MDA-MB-231 or vector-transfected control cells. However, the EWS-FLI1 probe did not hybridize to these products on Southern hybridization (Fig. 1*a*), indicating that they do not represent the EWS-FLI1 fusion transcript. The same membrane was then hybridized with the radiolabeled 22.4 EWS oligonucleotide primer, confirming transfer of the DNA visualized on the ethidium-stained gel to the membrane. The amplification pattern obtained when nested PCR was performed using the external 11.3 FLI1 and 22.3 EWS primers followed by the nested internal 11.4 FLI1 and 22.4 EWS primers was the same as that obtained after PCR with 11.4 FLI1 and 22.4 EWS (described above; data not shown). RT-PCR with the 11.3 FLI1 and 22.4 EWS primers amplified a 340-bp product in 231-EIA cells but not in the parental cells or vector controls. Again, Southern blotting was negative for these samples (Fig. 1*b*). Thus, although E1A is able to induce transcripts that can be amplified by primers derived from the *EWS* and *FLI1* genes, the E1A-induced transcripts do not represent the EWS-FLI1 fusion transcripts.

To confirm and understand what may cause false positive RT-PCR results in E1A-transfected cell lines, the RT-PCR products amplified from the 231-E1A cell line and the Ewing sarcoma line RD-ES were subcloned and sequenced. Sequencing of the RT-PCR product amplified from the RD-ES cell line with the 11.4 FLI1 and 22.4 EWS primers confirmed that it was the EWS-FLI1 fusion product (Fig. 1*c*). In contrast, sequencing of the bands from the 231-EIA transfectants (Fig. 1*a*) proved them to be unrelated genes; the 250-bp band corresponded to the mRNA for the human KIAA0477 protein, and the 130-bp band corresponded to the mRNA for the human KIAA0339 protein. The RT-PCR products were homologous to the fusion product EWS-FLI1 only in the primer sequences, with 100% homology in the 3′ ends of the 11.4 FLI1 and 22.4 EWS primers (Fig. 1*d*). The sequence of the 340-bp product amplified from the 231-E1A cell line with 11.3 FLI1 and 22.4 EWS primers (Fig. 1*b*) corresponded to that of human ribosomal protein L4 mRNA. Again, there was sequence homology between the 22.4 EWS primer and the L4 sequence (the L4 3′-untranslated region sequence annealing to the 11.3 primer is not available in GenBank for comparison). Thus, all of the amplified RT-PCR products in cell lines other than RD-ES, represented false positive results. These genes may be up-regulated with E1A transfection and deserve further study.

A total of 13 stable E1A transfectant E1A cell lines including MDA-MB-231, MCF-7, MDA-MB-435 (breast cancer), SKOV3-ip1 (ovarian cancer), and PC-3 (prostate cancer) were tested for the EWS-FLI1 fusion transcript using RT-PCR. The actual EWS-FLI1 fusion transcript could not be detected in any

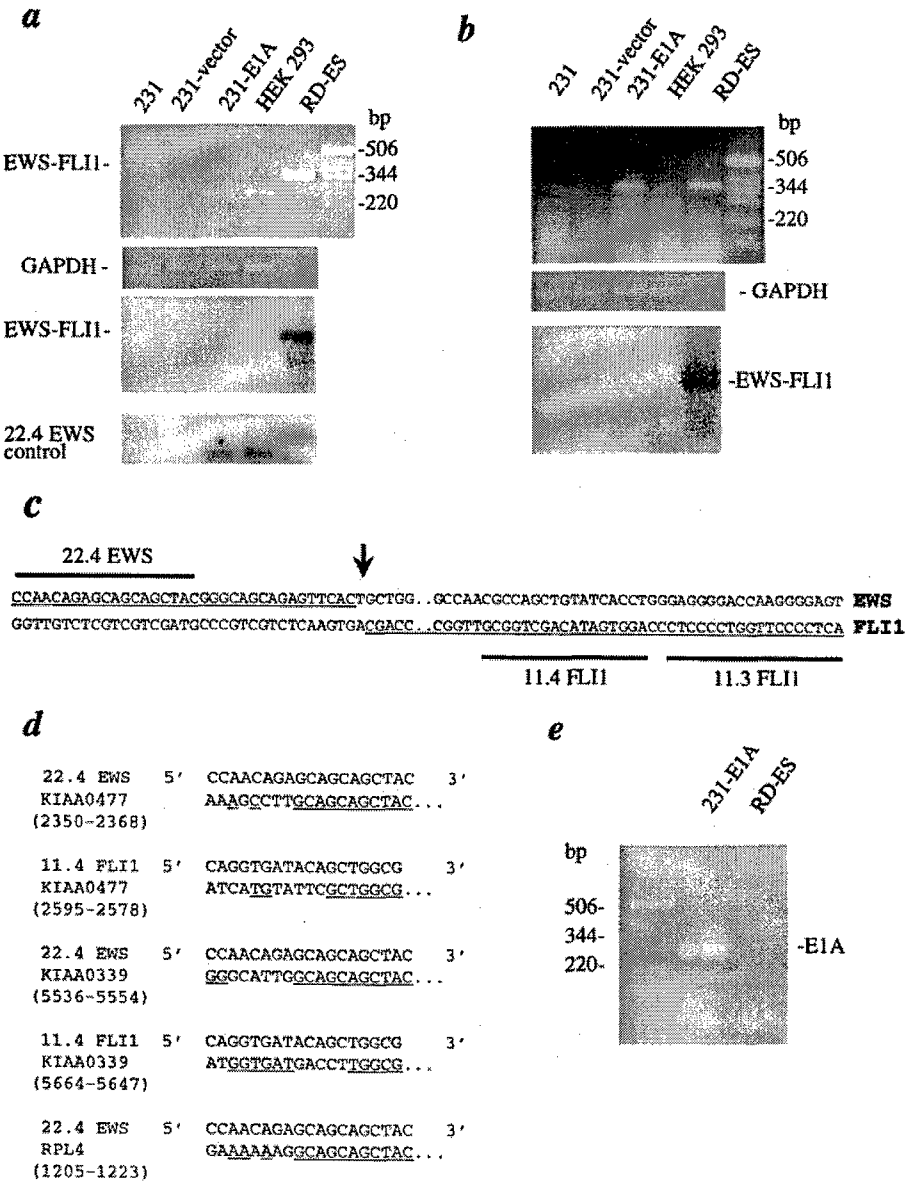

Fig. 1 RT-PCR and Southern blot hybridization of the fusion transcript EWS-FLI1. *a, top panel,* RT-PCR analysis with primers 11.4 FLI1 and 22.4 EWS on total RNA from MDA-MB-231 cells, vector-transfected 231 cells, E1A-transfected 231 cells, HEK 293 cells, and RD-ES Ewing sarcoma cells. *Upper middle panel*, RT-PCR of the same cell lines with primers for GAPDH as a control. *Lower middle panel*, Southern blot analysis of RT-PCR products shown in the *upper panel* with a EWS-FLI1 fusion product probe. *Bottom panel*, Southern blot of the same membrane with radiolabeled 22.4 EWS oligonucleotide probe as transfer control. *b, top panel,* RT-PCR analysis of RNA from the same cell lines with primers 11.3 FLI1 and 22.4 EWS. *Middle panel*, RT-PCR for GAPDH as a control. *Bottom panel*, Southern hybridization of RT-PCR products with the EWS-FLI1 probe. *c*, sequence corresponding to the EWS-FLI1 fusion transcript detected in the RD-ES cell line. The *arrow* indicates the breakpoint. RT-PCR using primers 22.4 EWS and 11.4 FLI1 would be expected to give a 344-bp product as detected in *a*; primers 22.4 EWS and 11.3 FLI1 would be expected to give a 363-bp product as detected in *b*. *d*, sequence alignment of the 22.4 EWS and 11.4 FLI1 primers with human mRNA for KIAA0477 protein (dbj, AB007946.1), human mRNA for KIAA0339 gene (dbj, AB002337), and human ribosomal protein L4 (RPL) mRNA (ref, NM 000986.1). Sequences with homology to the primers are *underlined*. The *numbers* in *parentheses* in the figure represent the location on the reference sequence used for alignment. *e*, RT-PCR analysis of RNA from 231-E1A and RD-ES cell lines with E1A primers.

of the cell lines except the RD-ES Ewing sarcoma line. RT-PCR was also performed with primers specific for E1A, and expression of the E1A transcript was confirmed in the E1A-transfected cell lines (data not shown). The E1A transcript was not detected by RT-PCR in the RD-ES Ewing sarcoma cell line (Fig. 1*e*). Thus, our study does not support the proposal by Sanchez-Prieta *et al.* (5) that E1A induces the Ewing tumor fusion transcript EWS-FLI1.

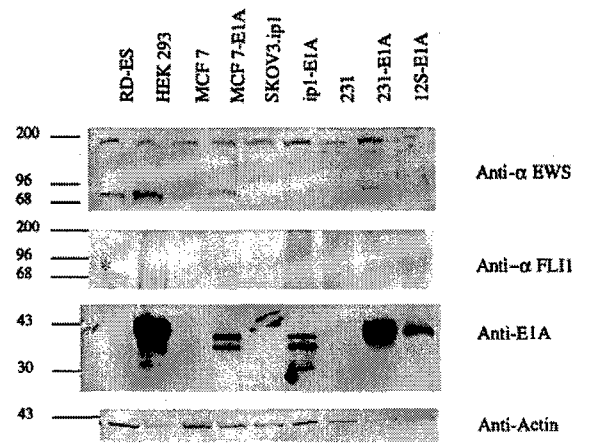

Fig. 2 Western blot analysis of EWS-FLI1 fusion proteins in different E1A stable cell lines. *Top panel*, Western blot analysis of cell lysates with an antibody against EWS protein. *Upper middle panel*, Western blot detection of the expression of EWS-FLI-1 fusion protein with a monoclonal antibody against FLI1. *Lower middle panel*, the same membrane was stripped and reprobed with a monoclonal antibody against E1A. *Bottom panel*, a parallel membrane was probed with a monoclonal anti-actin antibody.

E1A-transfected Cells Do Not Express the EWS-FLI1 Fusion Protein. The human breast cancer cell lines MCF-7 and MDA-MB-231 and ovarian cancer cell line SKOV3-ip1 were studied with and without transfection of E1A to detect the EWS-FLI1 fusion protein. The Ewing sarcoma cell line RD-ES was used as a control for the EWS-FLI-1 fusion protein. After immunoblotting with a polyclonal antibody against EWS; a M_r ~68,000 band was detected in all cell lines studied except SKOV3-ip1 and E1A-transfected ip1-E1A cells (Fig. 2). When a monoclonal anti-FLI1 antibody was used; the expected M_r 68,000 EWS-FLI1 fusion protein was detected only in the RD-ES cell line (Fig. 2). No fusion proteins were detected in any of the E1A-transfected cell lines. Immunoblotting with E1A antibodies confirmed the expression of the E1A protein in the E1A-transfected cell lines. The results indicate that the commercially available anti-EWS polyclonal antibody (Santa Cruz Biotechnology) also cross-reacts with a cellular protein about the same size (M_r ~68,000) as the EWS-FLI1 fusion protein. However, the M_r ~68,000 band detected in HEK 293, MCF-7, MCF-7-E1A, 231, and 231-E1A cells is not the EWS-FLI1 protein because it cannot be detected by the anti-FLI1 antibody.

Discussion

Sanchez-Prieto *et al.* (5) recently reported that adenovirus E1A induces the Ewing sarcoma fusion transcript EWS-FLI1. However, data from the studies of Melot and Delattre (6) and from Kovar *et al.* (7, 8) do not support this hypothesis. In our study, we examined 13 different cell lines stably transfected with E1A to test the hypothesis that expression of the *E1A* gene in human cells could elicit the specific fusion transcript EWS-FLI1. We were unable to find any evidence of the EWS-FLI1 fusion transcript or fusion protein in these E1A transfectants. Interestingly, we found expression of unrelated genes that may be induced by E1A, which can be detected under the PCR assay conditions used (Fig. 1, *a* and *b*). In addition, the commercially available anti-EWS antibody also cross-reacts with a cellular protein about the same size (M_r ~68,000) as the EWS-FLI1 fusion protein (Fig. 2). It is worthwhile to mention that the anti-EWS antibody-detected M_r ~68,000 protein is slightly enhanced in the E1A-transfected cells (compare MCF-7 with MCF7-E1A and 231 with 231-E1A). This phenomenon, without careful further examination with anti-FLI1 antibody to exclude its identity as the EWS-FLI1 fusion protein, could be easily but mistakenly used to support the notion that E1A induces the EWS-FLI1 fusion protein.

Adenovirus E1A has been examined in preclinical studies and found to be a promising therapeutic agent because of its abilities to suppress tumorigenesis and metastasis and to sensitize cells to apoptosis induced by cytotoxic agents (10–16). Extensive preclinical safety studies have been performed with E1A (17–19), and a Phase I clinical trial of *E1A* gene therapy for patients with advanced breast and ovarian cancer has recently been completed (3). Phase II studies of *E1A* gene therapy are currently in progress.

The proposal that E1A may induce EWS-FLI1 expression is revolutionary because it suggests that the Ewing tumor may be of viral etiology and that viral transfections may lead to chromosomal translocations. This hypothesis has implications regarding the safety of the use not only of E1A but also of adenoviral vectors containing E1A, such as Onyx-015 (20) in gene therapy trials. Our findings, however, support those of Melot and Delattre (6), and Kovar *et al.* (7, 8); we found no evidence for an association between E1A expression and expression of the EWS-FLI1 fusion product.

We have not studied the E1A integration site in the genome of our stable E1A transfectants. The integration site may be important for the activation of genes; however, Sanchez-Prieto *et al.* (5) have found that E1A-expressing HEK 293 and IMR 90 cells have multiple gene copies in many chromosomes, with no specific chromosomal site of integration. Thus, the site of gene integration is unlikely to account for differences in our results.

Although we cannot exclude the possibility that some E1A-expressing cell lines may have the EWS-FLI1 fusion as claimed by Sanchez-Prieto *et al.* (5, 9), the presence of such a fusion is not characteristic of E1A-transfected cells. We conclude that the potential for induction of such a gene rearrangement using *E1A* gene therapy is too low to be of clinical significance in the treatment of advanced malignant disease.

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Regulation of the Activity of p38 Mitogen-Activated Protein Kinase by Akt in Cancer and Adenoviral Protein E1A-Mediated Sensitization to Apoptosis

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The adenoviral early region 1A (E1A) protein mediates sensitization to different stimulus-induced apoptosis, such as tumor necrosis factor alpha, UV and gamma irradiation, and different categories of anticancer drugs. However, the molecular mechanisms underlying E1A-mediated sensitization to apoptosis are still not completely defined. Here, we show that E1A-mediated sensitization to apoptosis by the inactivation of a key survival factor Akt and the activation of a pro-apoptotic factor p38. Also, inactivation of Akt by either a specific inhibitor or a genetic knockout of Akt1 results in p38 activation, possibly through the release of the activity of p38 upstream kinases, including ASK1 and MEKK3. In addition, we showed that p38 phosphorylation is downregulated and Akt phosphorylation is upregulated in multiple human tumor tissues, and this correlates with tumor stage in human breast cancer. A deletion mutation of a conserved domain of E1A, which is required for E1A-induced downregulation of Akt activity, disrupts E1A-mediated upregulation of p38 activity and also eliminates E1A-mediated chemosensitization. Thus, activation of p38 and inactivation of Akt may have general implications for tumor suppression and sensitization to apoptosis.

Many types of tumors are associated with activated oncogenic kinases, and two complementary roles of these oncogenic kinases are stimulating signaling pathways that enable cells to function independent of their environment and making tumor cells resistant to genotoxic therapies, such as chemo- and radiotherapy (22, 24, 48). Deregulated growth signaling pathways and acquired resistance toward apoptosis therefore constitute two hallmarks of most, if not all, human tumors (18). For example, it has been shown that the serine/threonine kinase Akt and its family members Akt 2 and 3 are either amplified or their activity is constitutively elevated in human carcinomas such as breast, pancreatic, ovarian, brain, prostate, and gastric adenocarcinomas (39, 50). As it is a direct downstream target of phosphatidylinositol 3-kinase (PI3K), Akt is also a key oncogenic survival factor and can phosphorylate and inactivate a panel of critical proapoptotic molecules, including Bad, caspase 9, the Forkhead transcription factor FKHRL1 (known to induce expression of proapoptotic factors such as Fas ligand), GSK3-β, cell cycle inhibitors p21 and p27, and tumor suppressor TSC2, etc. (4, 25, 39, 50, 58). Akt can also inactivate p53, a key tumor suppressor, through phosphorylation and nuclear localization of MDM2 (33, 50, 59). Activation of Akt has been shown to induce resistance to apoptosis induced by a range of drugs (41). Thus, molecules that can block Akt activity may have important significance in cancer therapy and drug sensitization.

The adenovirus early region 1A protein (E1A) induces chemosensitization among different categories of anticancer drugs, including cisplatin, adriamycin, etopside, staurosporine, 5-fluorouracil, and paclitaxel (Taxol) (5, 14, 16, 32, 45, 53), suggesting that a general cellular mechanism may exist to regulate E1A-mediated chemosensitization. However, the molecular mechanisms underlying E1A-mediated chemosensitization are still not completely defined. Earlier studies on normal fibroblast cells revealed that E1A-mediated sensitization to cytotoxic anticancer drugs depends on the expression of functional p53 and p19ARF, an alternative splicing form of p16INK4a (12, 31, 32). E1A was also shown to downregulate Her-2/neu overexpression and facilitate E1A-mediated sensitization to the cytotoxicity of anticancer drugs in human breast and ovarian cancer cells (53, 55, 56). In another study, E1A was reported to mediate sensitization to anticancer drugs in human osteosarcoma cells (16) in a p53- and Her-2/neu-independent manner. Similarly, there is no correlation between p53 protein level and sensitivity of DNA-damaging agents in keratinocytes carrying adenovirus E1A (45). A few other critical molecules were also proposed to be involved in E1A-induced chemosensitization, such as the proapoptotic protein Bax, caspase 9, or a yet unidentified inhibitor that ordinarily provides protection against cell death (14, 15, 34, 43, 49, 52). However, none of the above molecules or pathways can really serve as a general cellular mechanism for E1A-mediated sensitization to apoptosis in a diverse cellular context. Recently, transcriptional upregulation of procaspases (such as pro-caspase 3, 7, 8, and 9) through E1A-mediated disruption of pRB function and subsequent release of free E2F-1 was reported to contribute to both p53-dependent and p53-independent drug sensitization by E1A in diploid normal fibroblast cells (37). In the present study, we found that E1A can activate p38 and inactivate Akt and showed that this pathway may provide a general cellular mechanism for E1A to mediate sensitization to different categories of anticancer drugs.

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MATERIALS AND METHODS

Cell culture, cell harvest, and Western blot. Human breast, ovarian, prostate, pancreatic, and colon cancer cell lines were grown in Dulbecco's modified Eagle's medium–F-12 (Life Technologies, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum. The stable E1A-expressing cell lines in breast cancer MDA-MB-231 and MCF-7 were established as described previously (36, 52). Similarly, domain deletion mutant constructs of E1A were transfected into MDA-MB-231 cells, and stable clones were screened and selected in the presence of G418. Akt1 knockout mouse embryonic fibroblasts (MEFs) and myristoylated, membrane-bound, constitutively active Akt1 (myr-Akt)-transfected stable Rat1 cells were provided by Nissim Hay (University of Illinois at Chicago, Chicago) (10).

For the analysis of basal Akt and p38 expression and activity, cells were serum starved overnight before harvesting. Cells were then washed twice with cold phosphate-buffered saline (PBS) and lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate ($NaVO_3$), and 1.5% aprotinin. The cell extracts were clarified by centrifugation, and protein concentrations were determined by using a Bio-Rad (Hercules, Calif.) protein assay reagent and analyzed in a spectrophotometer with bovine serum album (Sigma, St. Louis, Mo.) as the protein standard. Aliquots of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore Corp., Bedford, Mass.) by using standard procedures. The membranes were then subjected to Western blotting, and the blots were developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, N.J.).

Primary antibodies. For Western blot analysis, rabbit polyclonal antibodies against phospho-Akt (Ser 473, catalog no. 9271, 1:1,000 dilution) and phospho-p38 (Thr 180/Tyr 182, catalog no. 9211, 1:1,000 dilution) were purchased from Cell Signaling Technology (Beverly, Mass.). Rabbit polyclonal antibodies against nonphosphorylated total Akt (catalog no. 9272, 1:500 dilution), p38 (catalog no. 9212, 1:500 dilution), and cleaved poly(ADP-ribose) polymerase (PARP) (Asp214, catalog no. 9541, 1:1,000 dilution) were also from Cell Signaling Technology. A rabbit polyclonal antibody against beta-actin was used as a loading control for Western blotting and was purchased from Sigma. For immunohistochemical (IHC) study, we used an IHC-specific rabbit polyclonal antibody against phospho-Akt (Ser 473, catalog no. 9277) or an IHC-specific monoclonal antibody against phospho-p38 (Thr180/Tyr 182, catalog no. 9216) at a 1:75 dilution for both antibodies. The monoclonal antibody used against the E1A proteins was M58 (PharMingen, San Diego, Calif.). Rabbit polyclonal anti-Bax antibodies, a hamster anti-human Bcl-2 monoclonal antibody, and rabbit polyclonal anti-ASK1 (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Mouse monoclonal antibodies against human caspase 3 and 7 were from Transduction Laboratories (1:1,000 dilution, C31720; Lexington, Ky.) and BD PharMingen (1:1,000 dilution, 66871A), respectively. Rabbit polyclonal antibodies against human caspase 8 and 9 were from Santa Cruz Biotechnology (1:500 dilution, SC-7890/H-134) and Cell Signaling Technology (1:500 dilution, no. 9502), respectively. To detect hemagglutinin (HA)-tagged proteins, a monoclonal anti-HA antibody was used (1:1,000 dilution, catalog no. 1,583,816; Boehringer Mannheim, Indianapolis, Ind.). A monoclonal anti-FLAG antibody (M2) was purchased from Sigma (1:1,000 dilution).

Transient transfection, MTT assay, luciferase assay, and FACS analysis. The standard MTT assay was performed to measure the viable cells after treatment with anticancer drugs as described previously (52). Expression vectors for HA-p38, constitutively active Akt (CA-Akt), dominant-negative Akt (DN-Akt), and cytomegalovirus driving luciferase (pcDNA3-Luc) were used in this study. First, 10^5 cells in a 60-mm dish were transfected with 2.2 μg of total DNA by using the DC-Chol cationic liposome as described previously (52). After 48 h, the cells were split into three sets: one used for a luciferase assay after exposure with or without paclitaxel for 24 h, one used to analyze Akt and p38 protein expression, and one fixed in 75% ethanol, stained with propidium iodide (25 μg/ml), and sent for fluorescence-activated cell sorter (FACS) analysis. The percentage of paclitaxel-treated cells that exhibited luciferase activity was normalized by using the luciferase activity of the untreated cells as the baseline (100%). Standard deviations from three independent experiments were calculated.

Establishment of IPTG-inducible DN-p38 stable cell lines. One E1A-expressing MDA-MB-231 clone was cotransfected with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible DN-p38α construct (a gift from Philipp E. Schere, Albert Einstein College of Medicine, Bronx, N.Y.) and the plasmid pCMVLacI (Stratagene, La Jolla, Calif.). Stable clones were selected in the presence of 200 μg of hygromycin/ml.

Immunoprecipitation. After transient transfection with HA-tagged p38 or CA-Akt, cells were stimulated with 10 μM insulin for 15 min. Cells were then lysed, and cell lysates were centrifuged at 16,000 *g* for 30 min. The supernatants were then transferred to a fresh tube. Proteins were cleared via addition of a normal mouse or rabbit immunoglobulin G and immunoprecipitated with anti-p38, anti-Akt, or anti-HA antibodies. Immunoprecipitates were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Akt, p38, and ASK1 were detected by Western blotting.

Kinase assay. Nonradioactive kinase assay kits for p38 and Akt were purchased from Cell Signaling (New England BioLabs, Beverly, Mass.). The p38 and Akt kinase activities were measured according to the manufacturer's protocol with glutathione *S*-transferase (GST)–ATF-2 as the substrate for p38 and GST–GSK-3-β as the substrate for Akt.

Tissue microarray and immunohistochemistry. Tissue microarray slides (HistoArray no. IMH-343/BA2 and IMH-304/CB2) were purchased from IMGENEX (San Diego, Calif.). Detailed information about each slide is available online. Slide processing and immunohistochemical staining were performed according to the manufacturer's protocol. Briefly, tissue slides were heated at 60°C, deparaffinized in xylene, hydrated in graded ethanol, and then immersed in tap water. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 for 20 min in a 95°C water bath. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide solution followed by three sequential PBS washes (5 min each). Slides were then blocked by the respective normal serum for each primary antibody, incubated with primary antibody diluted in TBS-T (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween 20) containing 1% ovalbumin and 1 mg of sodium azide/ml, incubated with biotinylated secondary antibody for 30 min at room temperature, washed with PBS again, and incubated with avidin-biotin complex (Vestastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, Calif.). Slides were washed with PBS again, incubated with the AEC (3-amino-9-ethylcarbazole) substrate kit (catalog no. Sk-4200; Vector Laboratories, Inc.), and then counterstained in Meyer's xylene.

One representative slide per case was evaluated with the antibodies mentioned above. The intensities of staining seen in different areas of the same slide were analyzed according to criteria described previously in the literature (1). The intensity was designated 0 when no cells stained, 1+ when 10 to 20% of cells stained (weak), 2+ when 20 to 50% of cells stained (moderate), and 3+ when more than 50% of cells stained (strong).

Statistics. For statistical analysis, groups scored as 0 and 1+ were combined as weak staining while groups scored as 2+ and 3+ were combined as strong staining. Similarly, to simplify the statistical analysis, breast tumors with stages 1 and 2 were combined as early stages of tumors ($n = 25$ cases) while tumors with stages 3 and 4 were combined as late stages of tumors ($n = 25$ cases). Statistical analysis was performed by using χ^2 analysis.

RESULTS

E1A upregulates p38 activity and downregulates Akt activity. To determine whether apoptosis-related kinases are involved in E1A-mediated sensitization to apoptosis, we examined the phosphorylation status of three well-known kinases involved in regulation of apoptosis, p38, Akt, and JNK, in E1A-expressing MDA-MB-231 and MCF-7 cells (231-E1A and MCF-7-E1A) versus vector-transfected cells (231-Vect and MCF-7-Vect). We detected phosphorylated p38 in cells stably expressing E1A but not in vector-transfected cells. However, the level of phosphorylated Akt was much higher in vector-transfected cells than in E1A-expressing cells. The levels of total Akt and p38 were similar in both types of cells (Fig. 1A). Kinase assays showed that p38 activity was higher and Akt activity was lower in 231-E1A and MCF-7-E1A cells than in 231-Vect and MCF-7-Vect cells (Fig. 1B and C). We did not detect any difference in the level of phosphorylated JNK between E1A-expressing and vector-transfected cells (data not shown). These results indicated that E1A enhanced the activity of the proapoptotic kinase p38 and repressed the activity of the antiapoptotic kinase Akt but did not affect JNK phosphorylation.

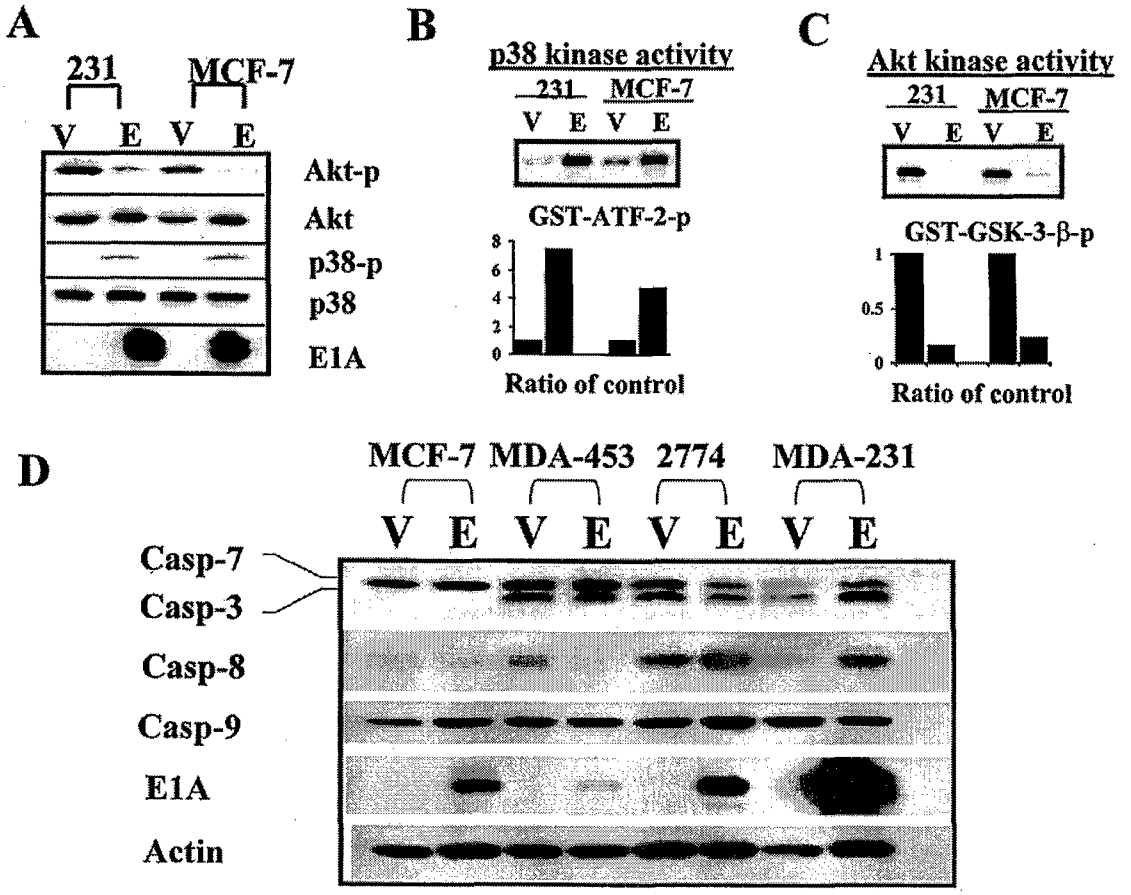


FIG. 1. Upregulation of p38 activity and downregulation of Akt activity by E1A correlated with E1A-mediated sensitization to paclitaxel-induced apoptosis. (A) Phospho-p38 (p38-p) and phospho-Akt (Akt-p) levels in E1A-expressing cells versus those in vector-transfected MDA-MB-231 and MCF-7 cells are shown. Total p38 (p38) and Akt (Akt) were used as loading controls. Results of kinase assays of p38 (B) and Akt (C) and densitometric analysis of relative p38 activity with GST–ATF-2 as a substrate and Akt activity with GST–GSK-3-β as a substrate in E1A-expressing cells versus vector-transfected control cells. E, E1A-expressing cells; V, vector transfected control cells. (D) Expression of caspase 3, 7, 8, and 9 proenzymes in E1A stable cells established in human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-453 and ovarian cancer cell line 2774. V, vector control; E, E1A stable cells.

A recent report showed that transient transfection of E1A resulted in the accumulation of caspase proenzymes in human normal diploid fibroblasts (37). We therefore compared the expression levels of caspase proenzymes caspase 3, 7, 8, and 9 between E1A transfectants and vector-transfected carcinoma cells, including breast cancer MDA-MB-231, MDA-MB-453, and MCF-7 cells and ovarian cancer 2774 cells. Unlike what was demonstrated with normal fibroblast cells, we did not observe a unanimous increase of these caspase proenzymes in the E1A stable cells established in human cancer cells with epithelial origins (Fig. 1D). This suggests that transcriptional upregulation of the caspase proenzymes in these human cancer cells may not be as critical as it is in the normal fibroblast cells and that other cellular mechanisms may exist for the E1A-mediated sensitization to apoptosis in human cancer cells.

Upregulation of p38 activity and downregulation of Akt activity correlate with E1A-mediated sensitization to paclitaxel-induced apoptosis. To test whether alteration of the kinase activity of Akt or p38 played a role in E1A-mediated sensitization to apoptosis, we compared the kinetics of phosphorylation of Akt or p38 with paclitaxel-induced apoptosis in 231-E1A cells by using PARP cleavage and Bcl-2 phosphorylation as apoptotic cell death markers. PARP cleavage and Bcl-2 phosphorylation occurred after decreased Akt phosphorylation and increased p38 phosphorylation in 231-E1A cells after exposure to 0.01 μM paclitaxel (Fig. 2A). However, no significant change was detected in the protein levels of p53 and Bax (Fig. 2A). The same concentration of paclitaxel did not trigger PARP cleavage, induce Bcl-2 phosphorylation, or modulate the levels of phosphorylated p38 and Akt in the parental MDA-MB-231 cells (data not shown). To trigger a similar response in parental MDA-MB-231 cells, a much higher dosage was required (Fig. 2B). The results suggest that downregulation of Akt and upregulation of p38 activities may be involved in the E1A-mediated sensitization to paclitaxel-induced apoptosis.

Activation of p38 and inactivation of Akt are required for E1A-mediated sensitization to drug-induced apoptosis. To

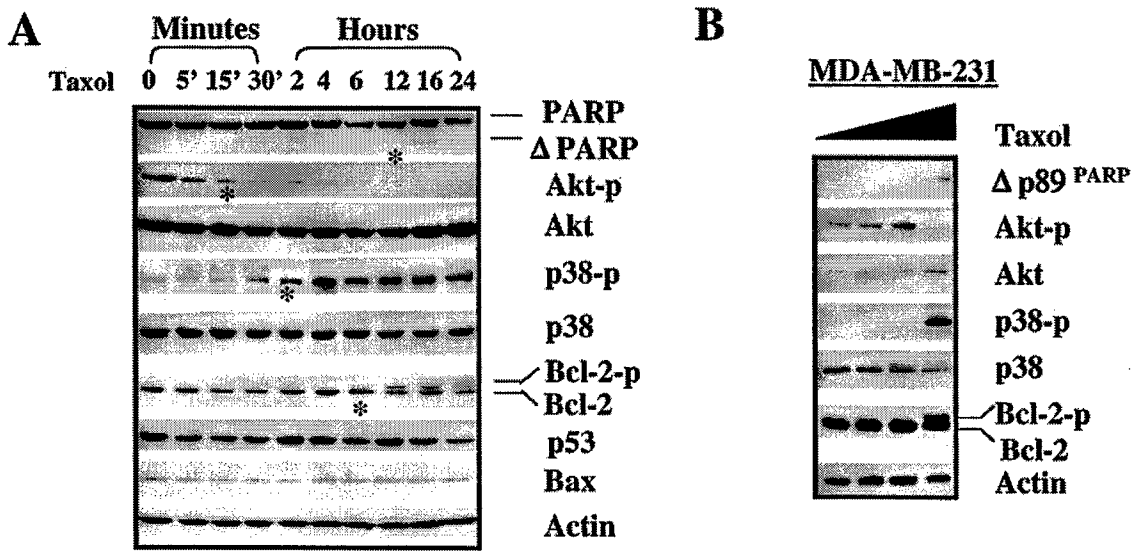


FIG. 2. Upregulated p38 activity and downregulated Akt activity correlate with E1A-mediated sensitization to paclitaxel-induced apoptosis. (A) Kinetics of PARP, Akt, p38, Bcl-2, p53, and Bax protein expression in 231-E1A cells before and after exposure to 0.01 μM paclitaxel. Bcl-2 phosphorylation and PARP cleavage were detected at 6 and 12 h after exposure to paclitaxel and became more obvious thereafter. Phosphorylation of p38 was first detected after 30 min to 2 h and then become more obvious after 4 h of treatment. Dephosphorylation of Akt could be detected even earlier, at 5 min posttreatment. Actin was used as a loading control. A dramatic alteration of each molecule was marked with an asterisk. (B) Dose-dependent effect of PARP cleavage and Akt and p38 phosphorylation in MDA-MB-231 cells after exposure to paclitaxel for 24 h. The concentrations of paclitaxel used ranged from 0 to 0.001, 0.01, and 0.1 μM.

evaluate whether activation of p38 is required for E1A-mediated sensitization to paclitaxel, we tested whether blocking p38 activity could inhibit E1A-mediated sensitization in 231-E1A cells. We used the specific p38 inhibitor SB203580 (Fig. 3A) and a DN-p38 mutant to block p38 activation (Fig. 3B). A pcDNA3-Luciferase (pcDNA-Luc) construct was transfected into 231-E1A cells, and luciferase activity was used as a measurement for cell survival. Pretreatment with SB203580 inhibited the phosphorylation of p38 in cells with or without exposure to paclitaxel (Fig. 3C, lanes 3 to 4) and protected cells from a paclitaxel-induced decrease of luciferase activity (Fig. 3C, lane 2 versus lane 4). In addition, FACS analysis showed that pretreatment with SB203580 protected 231-E1A cells from paclitaxel-induced apoptosis (27.5% versus 18.0%) (Fig. 3D, lanes 2 and 4, bottom). These data suggest that p38 activation is required for E1A-mediated sensitization to paclitaxel-induced apoptosis. Using a DN-p38 to block p38 activation further supported the above results (Fig. 3B). When the cells were switched to medium containing 5 μM IPTG for 24 h, expression of IPTG-inducible DN-p38 was induced in the presence or absence of paclitaxel (Fig. 3B, lower panel). FACS analysis showed that induction of DN-p38 by IPTG significantly inhibited paclitaxel-induced apoptosis (Fig. 3B, upper panel). Identical results were obtained when two additional stable clones were studied (data not shown). However, IPTG could not induce this effect in the 231-E1A cells without IPTG-induced DN-p38 (data not shown). Taken together, these data suggest that p38 activation is required for E1A-mediated sensitization to paclitaxel-induced apoptosis.

To determine whether downregulation of Akt activity is also required for E1A-mediated sensitization to paclitaxel, we examined whether activation of Akt by transfection of CA-Akt would inhibit paclitaxel-induced apoptosis in 231-E1A cells. The level of phosphorylated Akt and luciferase activity was increased in CA-Akt-transfected 231-E1A cells compared with that in the control 231-E1A cells (Fig. 3C, lanes 1 and 2 versus lanes 5 and 6). FACS analysis showed that fewer apoptotic cells were detected in CA-Akt-transfected cells (15.9%) than in control 231-E1A cells (27.5%) after exposure to paclitaxel (Fig. 3D, lane 2 versus lane 6, bottom). Thus, inhibition of Akt phosphorylation is also required for E1A-mediated sensitization to paclitaxel-induced apoptosis.

Activation of p38 and inactivation of Akt represent a general cellular mechanism in response to different apoptotic stimuli. To determine whether the same mechanism of E1A-mediated sensitization to paclitaxel applies to other anticancer drugs, we tested the effects of four additional drugs used in the clinic for treatment of human cancer. These four anticancer drugs induce antitumor activities through different modes of action: doxorubicin-adriamycin (topoisomerase II inhibitor), cisplatin (DNA-damaging agent), methotrexate (antimetabolite drug), and gemcitabine (antimetabolite drug). The expression of E1A significantly enhanced each drug's cytotoxicity in MDA-MB-231 cells, determined by using the MTT assay (Fig. 4A). In addition, downregulation of Akt phosphorylation and upregulation of p38 phosphorylation and PARP cleavage were observed in 231-E1A cells but not in 231-Vect cells treated with each of the drugs at the same dosage (Fig. 4B). These results suggest that activation of p38 and inactivation of Akt may contribute to E1A-mediated sensitization to apoptosis induced by these different drugs.

To address whether activation of p38 and inactivation of Akt

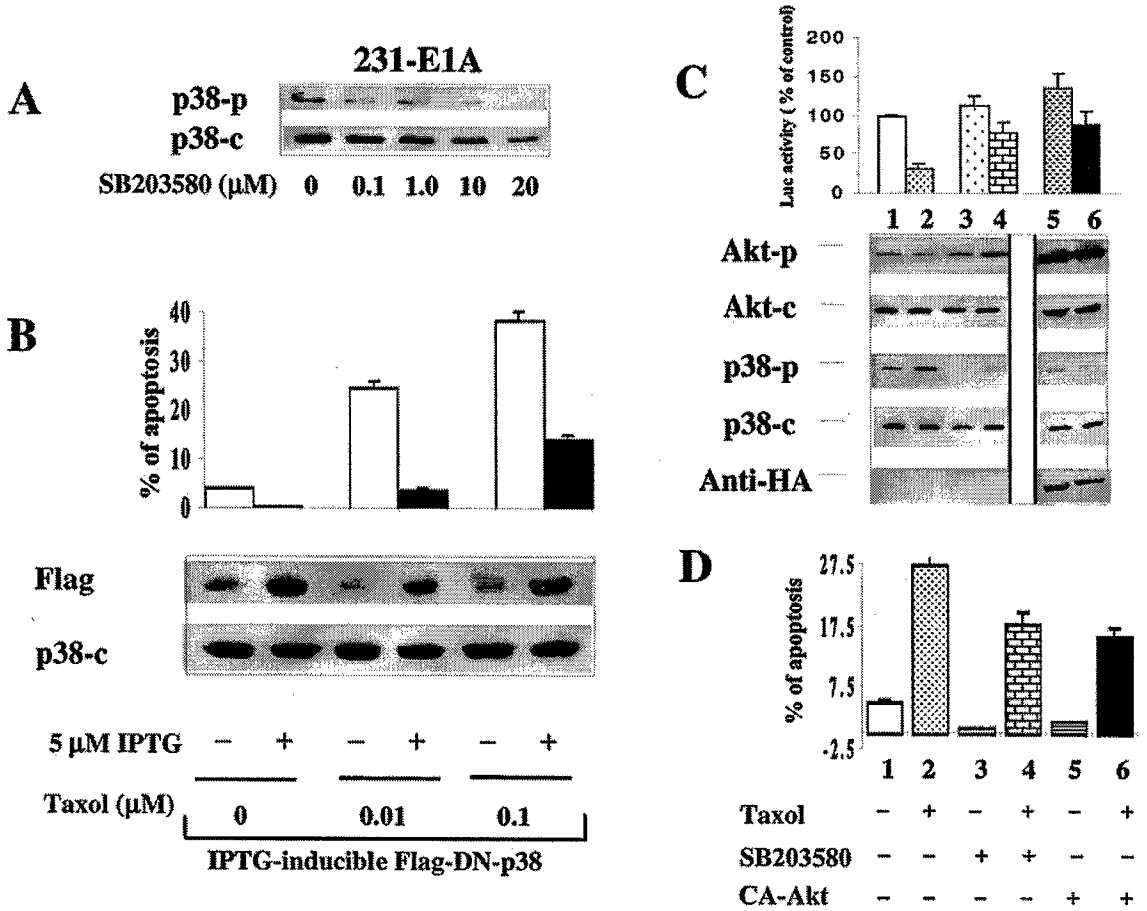


FIG. 3. Activation of p38 and inactivation of Akt are required for E1A-mediated sensitization to paclitaxel-induced apoptosis. (A) Dose-dependent effect of SB203580 on p38 phosphorylation in 231-E1A cells. (B) An IPTG-inducible, Flag-tagged DN-p38 stable cell clone was established in 231-E1A cells. Repression of p38 activity by IPTG-inducible DN-p38 enhances Akt phosphorylation and eliminates E1A-mediated sensitization to paclitaxel in E1A-expressing cells in the presence of 5 μM IPTG for 24 h. (C) Western blot analysis of p38 and Akt in E1A-expressing MDA-MB-231 cells and luciferase assay. The viability of cells with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) exposure to paclitaxel was measured by luciferase assay. The pcDNA3-Luc vector was cotransfected into 231-E1A cells with (lanes 5 and 6) or without a HA-tagged, myristoylated, membrane-targeted CA-Akt (lanes 1 to 4) before treatment with paclitaxel. After exposure to paclitaxel for 4 h, a portion of cells was harvested for protein extraction while the rest were grown for 24 h. In the absence of 20 μM SB203580, the level of phosphorylated p38 increased after exposure to paclitaxel (lanes 1 and 2). Expression of CA-Akt was detected with an anti-HA monoclonal antibody (lanes 5 and 6). (D) A portion of the above-described cells was also subjected to FACS analysis to measure apoptosis. +, present; −, absent.

also applied to drug-induced apoptosis in the absence of E1A, we tested whether increasing the dosage of gemcitabine or adriamycin could also enhance p38 activation and inhibit Akt activation, which would then contribute to drug-induced apoptosis. When MDA-MB-231 and HBL-100 cells were exposed to a dose of gemcitabine or adriamycin 10 times higher than that used in experiment whose results are shown in Fig. 4B, we observed a similar pattern of downregulation of Akt and upregulation of p38 activation, which was correlated with PARP cleavage (Fig. 4C). Similar results were also observed in MDA-MB-231 cells when exposed to a higher dose of paclitaxel (Fig. 2B). These results suggest that p38 activation and Akt inactivation may not be limited to E1A-mediated sensitization to apoptosis but may also contribute to drug-induced apoptosis in the absence of E1A. Thus, downregulating Akt activity and upregulating p38 activity may represent a general cellular mechanism of response to apoptotic stimuli, and E1A may turn on this cellular mechanism and mediate sensitization to drug-induced apoptosis.

To determine the physiological relevance of inactivation of Akt and activation of p38 in the execution of apoptosis, we extended our investigation to apoptosis induced by serum starvation, tumor necrosis factor alpha (TNF-α), and UV irradiation. We observed that phosphorylation of p38 and dephosphorylation of Akt were correlated with serum starvation-, TNF-α-, and UV-induced PARP cleavage in 231-E1A cells, especially in detached apoptotic cells (Fig. 4D, lanes 1 to 8). However, a dose of 10 times higher is required for inducing a response in parental MDA-MB-231 cells similar to that in 231-E1A cells (Fig. 4D, lanes 9 to 11). Taken together, down-

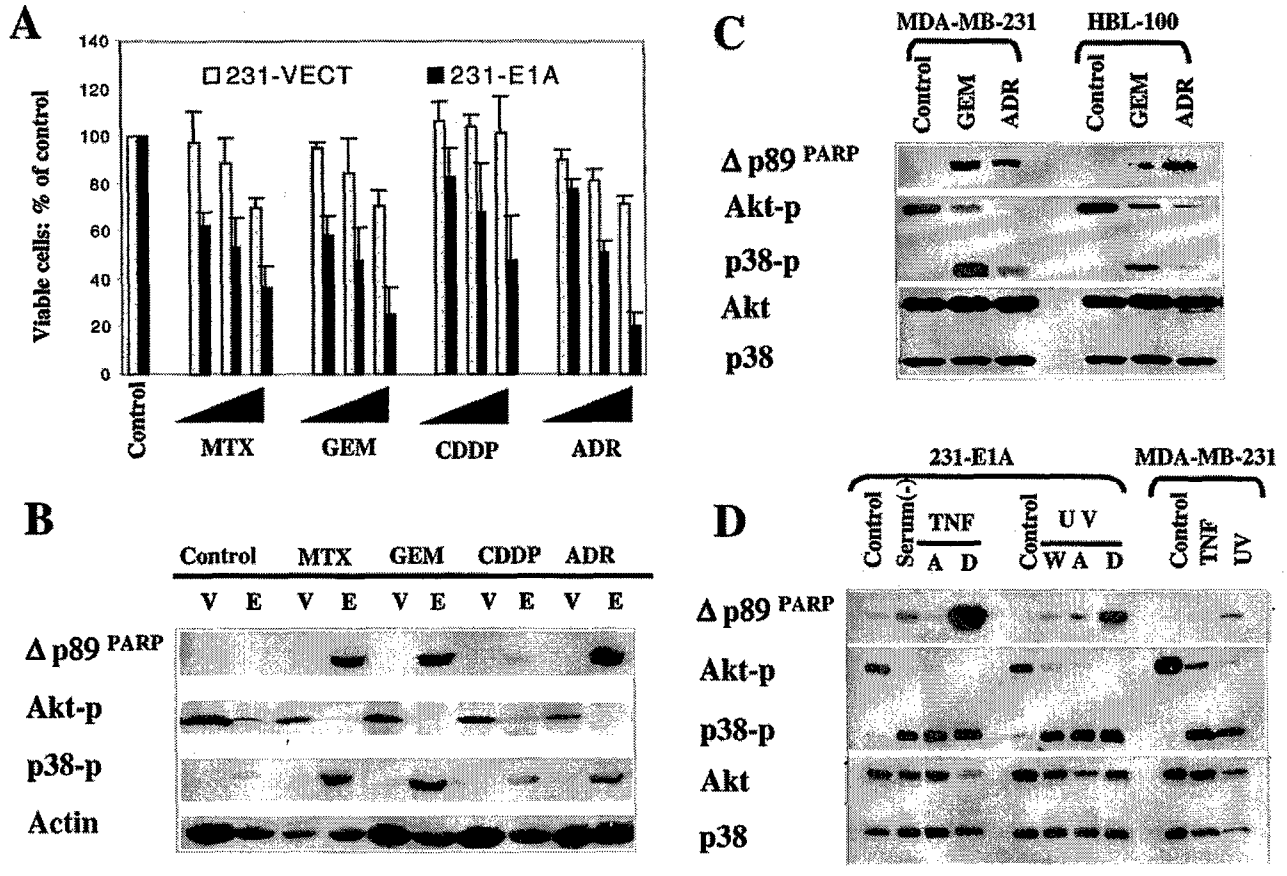


FIG. 4. Activation of p38 and inactivation of Akt represent a general cellular mechanism in response to different apoptotic stimuli. (A) Percentage of viable cells in vector-transfected (231-Vect) and E1A-expressing MDA-MB-231 (231-E1A) cells after exposure to different doses of adriamycin (ADR) (0.1, 1.0, and 10 μM), cisplatin (CDDP) (0.2, 2, and 10 μg/ml), gemcitabine (GEM) (0.2, 2, and 10 μg/ml), and methotrexate (MTX) (0.2, 2, and 10 μM) for 24 h. Cell viability was measured by using the MTT assay. (B) Downregulation of Akt activation and upregulation of p38 activation correlated with drug-induced PARP cleavage in 231-Vect (V) and 231-E1A (E) cells. The concentrations used were 1 μM ADR, 2-μg/ml CDDP, 2-μg/ml GEM, and 2 μM MTX. (C) The concentrations of GEM and ADR used for MDA-MB-231 and MCF-7 cells were 20 μg/ml and 20 μM, respectively. (D) Downregulation of Akt and upregulation of p38 phosphorylation correlated with PARP cleavage induced by serum starvation, TNF-α, and UV irradiation. 231-E1A cells were serum starved [serum(−)], exposed to TNF-α (5 ng/ml), or UV irradiated (6 J/cm^2) while parental MDA-MB-231 cells were exposed to 10-times-higher doses of TNF-α (50 ng/ml) and UV radiation (60 J/cm^2). Both the attached cells and cells in suspension were collected, if not specified. W, whole-cell lysate with both attached and suspended cells; A, attached cells only; D, detached or floated apoptotic cells.

regulation of Akt activation and upregulation of p38 activation may also represent a general cellular mechanism in response to different apoptotic stimuli.

The physiological regulation of p38 activity by Akt is through ASK1 and MEKK3, the upstream kinases of p38. The above results suggest that both downregulation of Akt and upregulation p38 activities are involved in E1A-mediated sensitization to apoptosis. We noticed that reduced Akt phosphorylation occurs before enhanced p38 phosphorylation in the kinetic study of E1A-mediated sensitization to paclitaxel-induced PARP cleavage (Fig. 2A). We therefore asked whether Akt may act upstream of p38. To this end, Akt activity was blocked by either a specific PI3K inhibitor, wortmannin, or a genetic method to knock out Akt expression. Blocking Akt activation with wortmannin in MDA-MB-231 cells resulted in decreased Akt phosphorylation and increased p38 phosphorylation (Fig. 5A). And when Akt phosphorylation was recovered, the p38 phosphorylation was reduced again (8- to 24-h time points). These results indicate that Akt phosphorylation was required for repressing p38 activation, suggesting that the former is upstream from the latter. This conclusion was further supported by the study with Akt1-knockout MEFs and myr-Akt1-transfected stable cells. We observed that the level of phosphorylated p38 was increased in Akt1$^{-/-}$ MEFs compared with that in Akt$^{+/+}$ and Akt$^{+/-}$ MEFs. Furthermore, the phospho-p38 protein was undetectable in the Akt constitutively activated myr-Akt1 stable cells (Fig. 5B). These results indicate that Akt is able to inhibit p38 activity.

In an attempt to determine how Akt regulates p38, we sought to determine whether Akt is physically associated with p38 by using coimmunoprecipitation experiments. We did not detect p38 in immunoprecipitated Akt samples (Fig. 5C) or Akt in immunoprecipitated p38 samples (data not shown), suggesting that Akt and p38 were not directly associated under

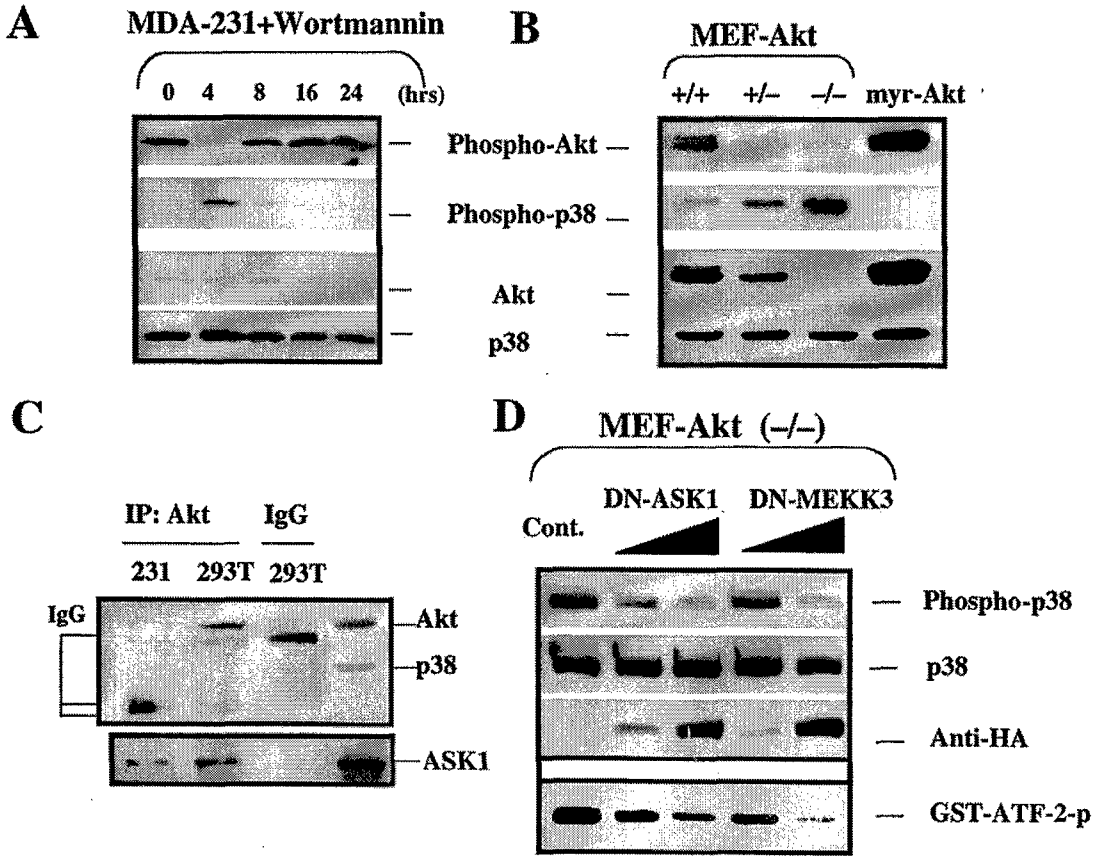


FIG. 5. Physiological regulation of Akt and p38 pathways. (A) Stable E1A-expressing or parental MDA-MB-231 cells were serum starved for 24 h before exposure to 20.0 μM 0.1 μM wortmannin. (B) Expression of phospho-p38 and phospho-Akt in Akt1 knockout MEFs and myr-Akt-transfected Rat1cells. (C) CA-Akt was transiently transfected into both MDA-MB-231 and 293T cells. The cells were lysed after transfection, and Akt was immunoprecipitated (IP). Western blot analyses of Akt, ASK1, and p38 interaction in 293T cells and MDA-MB-231 cells were performed. (D) $Akt^{-/-}$ MEFs were grown in six-well plates for 24 h and then transiently transfected by FuGENE 6 liposome (catalog no. 1 814 443; Roche Molecular Biochemicals, Indianapolis, Ind.) at a 3:1 ratio with either HA-tagged DN-ASK1 or HA-tagged DN-MEKK3 cDNA in the amounts of 1 and 10 μg, respectively. Cells were grown for another 36 h and were then harvested and analyzed for p38 kinase activity with ATF-2 as the substrate. The expressions of phospho-p38, total p38, and the HA tag were also detected by the respective antibodies. IgG, immunoglobulin G; Cont., control cells transfected with pcDNA3 plasmid DNA.

the conditions we used. A recent report demonstrated that ASK1 is a substrate of Akt (28), and ASK1 has been shown to be an upstream kinase of p38 (23, 51), suggesting that Akt may indirectly regulate p38 activity through ASK1. Indeed, we also detected that ASK1 was coimmunoprecipitated with Akt in our experimental system (Fig. 5C). To test whether Akt can down-regulate p38 activation through the repression of p38 upstream kinases, such as ASK1, we blocked the activity of either ASK1 or MEKK3, both of which are p38 upstream kinases that can be inactivated by Akt (17, 28), by using a kinase-dead, DN mutant of ASK1 (DN-ASK1) or MEKK3 (DN-MEKK3). As expected, blockade of either ASK1 or MEKK3 activity by DN-ASK1 or DN-MEKK3 repressed p38 phosphorylation and its kinase activity, as measured by phosphorylation of ATF2 in a dose-dependent manner in $Akt1^{-/-}$ MEFs (Fig. 5D), suggesting that Akt inhibits p38 activation through repression of ASK1 and/or MEKK3 activation.

p38 inactivation is associated with Akt activation in human cancer. The above results suggest that Akt acts upstream of p38 and blocks p38 activation. Because activation of Akt is a common phenomenon in different types of human cancers, we asked whether p38 inactivation is also a common phenomenon in human cancer cells and correlates with Akt activation. To test whether p38 inactivation was accompanied by Akt activation in human tumor tissues in vivo, we utilized tissue array slides to screen phospho-p38 and phospho-Akt expression in tumor tissues of different origins and normal or parallel normal organ tissues. We found that the phospho-Akt level was dramatically higher while phospho-p38 was undetectable in most of the cancer tissues obtained from different types of solid tumors, such as breast, lung, liver, bile duct, gastric, colorectal, renal cell, ovarian, and uterine cancers; malignant lymphoma; and Schwannoma. In contrast, the intensity of phospho-p38 protein staining was relatively strong while that of phospho-Akt staining was very weak in normal organs and parallel healthy tissues. Representative data on the expression of phospho-p38 and phospho-Akt in healthy versus tumor tissues obtained from the breast, lung, liver, and biliary duct are shown

in Fig. 6A. By screening a panel of breast, ovarian, prostate, pancreatic, and colorectal cancer cell lines with phosphospecific antibody against p38 or Akt, we also observed a correlation between enhanced Akt phosphorylation and reduced p38 phosphorylation in these human cancer cell lines (data not shown). These data support the hypothesis that p38 activity is repressed in different types of human cancer, which is associated with enhanced Akt activation.

To test whether repression of p38 and activation of Akt also correlate with tumor stage in human cancer, we analyzed 10 healthy breast tissue samples (including 2 healthy nipple and 8 healthy breast tissues) and 50 cases of breast cancer at different stages on Histo-Array slides. These include 4 cases at stage I (T1), 21 cases at stage II (T2), 20 cases at stage III (T3), and 5 cases at stage IV (T4). Among the 25 cases of early stage tumors (T1 and T2), 12% of them are negative and half of them are weakly positive for phospho-Akt staining. In the advanced late-stage tumor samples, all of them are positive and more than 80% of them are moderately to strongly positive for phospho-Akt staining (Fig. 6B), whereas the relative intensity of phospho-p38 staining was inversely correlated with the tumor stage (Fig. 6B). In the early stage tumor samples, only one-third of them (36%) are positive for phospho-p38 staining and only 1 of 25 cases is moderately positive for phospho-p38. In the late-stage tumor samples (T3 and T4), 80% of them are negative for phospho-p38 staining and the rest are weakly positive for phospho-p38 staining (Fig. 6B). Comparing the phospho-p38 staining and phospho-Akt staining in the advanced late-stage tumor samples, we observed an inverse correlation between the intensity of strong phospho-Akt staining versus weak phospho-p38 staining ($P < 0.0001$) (Fig. 6C). Phosphorylated p38 could be detected in most of the healthy organ tissues but not in most of the cancer tissues or cell lines, indicating that p38 inactivation is also a common event in human cancer cells with Akt activation.

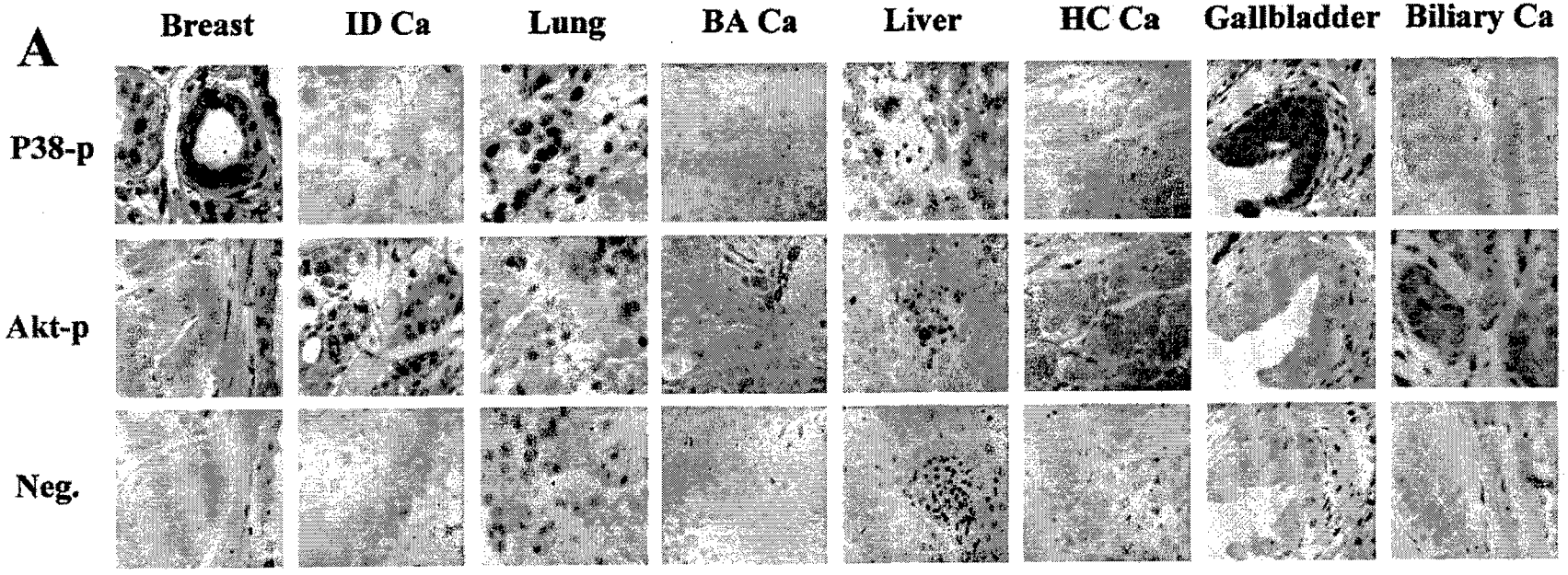
The CR2 domain of E1A is required for downregulation of Akt phosphorylation and chemosensitization. The above results suggested that Akt regulated p38 activation in both physiological and pathological conditions, which indicates that E1A-mediated downregulation of Akt activity and upregulation of p38 activity are accompanied events, i.e., by repression of Akt activation, E1A enhanced p38 activity and sensitized cells to drug-induced apoptosis. To lay further genetic support for these conclusions, we proposed to map the domain(s) of E1A that is responsible for downregulation of Akt activity and demonstrate that the same domain is also critical for E1A-mediated upregulation of p38 and sensitization to drug-induced apoptosis. It is known that among the three conserved domains (CR) of E1A, CR1 and CR2 are associated with E1A-mediated sensitization to apoptosis (46). Therefore, we established E1A functional domain deletion mutation stable cells in MDA-MB-231 cells, including wild-type E1A, and deletion mutations of CR1 (ΔCR1) and CR2 (ΔCR2) (Fig. 7A). We found that deletion mutations of the CR2 domain dramatically disrupted E1A's ability to downregulate Akt kinase activity, eliminated E1A-mediated upregulation of p38 kinase activity, and remarkably repressed E1A-mediated sensitization to paclitaxel-induced apoptosis (Fig. 7B and C) while the CR1 domain mutant only slightly affected E1A-mediated chemosensitization and downregulation of Akt and upregulation of p38 activities (Fig. 7B and C). These results indicate that the same CR2 domain required for downregulation of Akt is also required for upregulation of p38 and sensitization to drug-induced apoptosis and thus supports the conclusions that Akt represses p38 activity and that E1A, by downregulation of Akt activity, enhances p38 activation and sensitizes cells to anticancer drug-induced apoptosis.

DISCUSSION

The present study shows that the activity of p38 is regulated by Akt and is deregulated partly due to Akt activation in human cancer. Activation of Akt antagonizes p38 activation while inactivation of Akt results in p38 activation. The adenoviral protein E1A, by downregulation of Akt activity, enhanced p38 activation and sensitized cells to apoptosis induced by different apoptotic stimuli. It is known that p38 participates in the regulation of apoptotic cell death through transcriptional upregulation of proapoptotic gene expression, such as Fas ligand (11, 13, 20, 38). p38 is also involved in negative regulation of cell growth, as it represses cyclin D1 expression and regulates the G_2-M transition through the regulation of cdc25 protein phosphatase and p53 protein (6, 42). Recently, inactivation of p38 has been shown to contribute to the development of human cancers by suppressing p53 activation (7), suggesting a tumor-suppressive function of p38. In contrast, Akt is known to upregulate the cyclin D1 expression while repressing Fas ligand expression and p53 stabilization (39, 50). Akt is also involved in regulation of the G_2-M transition (26, 40, 47). Thus, Akt may functionally antagonize the p38 effect on cellular processes ranging from cell cycle progression to cell death, though some cell types may respond differently (3, 8, 17, 19, 44). However, the regulation between the Akt and p38 pathways is still unclear in the literature, and the present study provides a link between activation of Akt and inactivation of p38. As discussed above, Akt positively regulates cell growth but negatively regulates cell death while p38 positively regulates cell death but negatively regulates cell growth. Given the results we obtained in $Akt^{-/-}$ MEFs (Fig. 5B and D) and the fact that Akt directly phosphorylates and negatively regulates the activation of ASK1 and MEKK3, the upstream kinase of p38 (17, 28), we propose that Akt may repress p38 activation through the phosphorylation and inactivation of ASK1 or MEKK3 and that inactivation of Akt may result in p38 activation through the release of ASK1 and/or MEKK3 activity. Because either DN-ASK1 or DN-MEKK3 sufficiently repressed enhanced p38 phosphorylation in $Akt1^{-/-}$ MEFs (Fig. 5D), it also suggests that both ASK1 and MEKK3 are involved in Akt-mediated inactivation of p38. Regulation of p38 activity by Akt2 through ASK1 was also demonstrated recently by Yuan et al. in their report on cisplatin-induced apoptosis (57), suggesting that both Akt1 and Akt2 may use a similar mechanism to inactivate p38.

We have shown that activation of p38 follows inactivation of Akt (Fig. 2 and 4B to D) when cells underwent apoptosis, suggesting that the pro- and antiapoptotic signals may integrate each other to prepare cells to commit suicide. The relative Akt and p38 activity may determine a cell's response to apoptotic stimuli, as they can be observed in E1A-mediated sensitization to apoptosis induced by serum starvation, TNF-α,

A



6844

B

Immunohistochemistry analysis of phospho-Akt and phospho-p38 expression in human breast cancer tissues

	Phospho-Akt		Phospho-p38	
T Stage	T1~T2 (n=25)	T3~T4 (n=25)	T1~T2 (n=25)	T3~T4 (n=25)
–	3 /25 (12%)	0 /25 (0)	18/25 (64%)	20/25 (80%)
+	11/25(44%)	4 /25 (16%)	8 /25 (32%)	5 /25 (20%)
++	6 /25 (24%)	11/25(44%)	1 /25 (4%)	
+++	6 /25 (24%)	10/25(40%)		

C

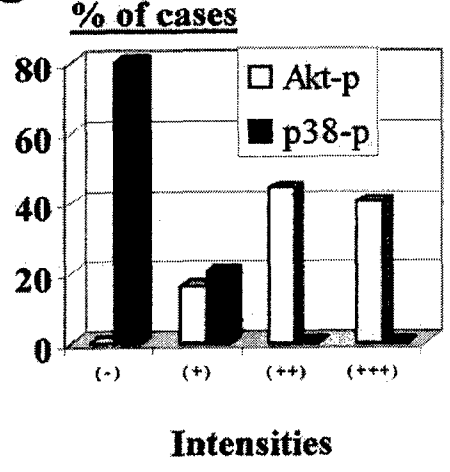


FIG. 6. Inactivation of p38 associated with Akt activation in human cancer. (A) Immunohistochemical staining of phospho-p38 and phospho-Akt in different types of human cancer tissues in the Histo-Array slides. Ca, carcinoma; ID Ca, infiltrating ductal carcinoma; BA Ca, bronchoalveolar carcinoma; HC Ca, hepatocellular carcinoma. (B) Intensity of phospho-Akt and phospho-p38 stained in the early and late stages of breast cancer. The staining intensity of phospho-Akt is significantly greater in late stage (stage III and IV) breast cancer while positive phospho-p38 staining is predominant in healthy breast epithelial cells and early stage (stage I and II) breast cancer ($P < 0.001$ for both). (C) Inverse correlation between phospho-Akt and phospho-p38 staining in late stage breast cancer tissue samples.

UV irradiation, and different categories of chemotherapeutic drugs (Fig. 4B to D). Expression of E1A may shift the balance of the pro- and antiapoptotic signals by repressing Akt activity and enhancing p38 activity, thereby favoring the proapoptotic signal. Additional approaches could also be used by E1A to shift the intracellular signal integration to favor a proapoptotic signal, such as activation of p53 and caspase proenzymes, but these pathways may not contribute to the present study. For example, p53 and p14ARF were deleted in MDA-MB-231 cells and we did not detect any change in the expression level of p53 or p14ARF in E1A versus parental control cells (data not shown), i.e., the p53-dependent mechanisms may not contribute to E1A-mediated sensitization to apoptosis in the present study. Although expression of E1A by infection of cells with either retroviral or adenoviral vector resulted in the accumulation of caspase proenzymes, such as caspase 3, 7, 8, and 9, by a direct transcriptional mechanism through enforced E2F-1 release in normal diploid human fibroblasts (IMR90) (37). We did not observe a consistent increase of these caspase proenzymes in the E1A stable cells established in human cancer cells with epithelial origin (Fig. 1D). In addition, sensitization to the DNA damage agent adriamycin-induced apoptosis by E1A is dependent on p53 status in normal fibroblasts (37) while E1A dramatically sensitized the adriamycin therapeutic effect in ovarian cancer SKOV3.ip1 (5) and breast cancer MDA-MB-231 cells (Fig. 4A), which do not express functional p53. The discrepancy between normal diploid fibroblasts and epithelial carcinoma cells in E1A-mediated sensitization to apoptosis may reflect the nature of the intrinsic difference between normal fibroblasts and carcinoma cells. However, downregulation of Akt activity by E1A was also observed in E1A-mediated sensitization to cisplatin in human normal IMR90 fibroblasts (54). Thus, targeting the key oncogenic survival factor Akt may represent a critical mechanism for E1A-mediated sensitization to anticancer drug-induced apoptosis in human cancer cells and normal fibroblasts as well.

E1A has been shown to facilitate cytochrome *c* release from the mitochondria, which also contributes to E1A-mediated sensitization to anticancer drugs. However, the mechanism by which E1A facilitates cytochrome *c* release is unclear (14). Although we did not test whether E1A expression affected Bax translocation, which may also facilitate cytochrome *c* release, E1A expression or treatment with paclitaxel did not affect the levels of Bax protein in our system (Fig. 2A). Akt is known to play an important role in maintaining mitochondrial integrity and inhibiting the release of cytochrome *c* (11, 27). Overexpression of Akt confers resistance to paclitaxel by inhibiting paclitaxel-induced cytochrome *c* release (41). However, p38 is also involved in regulation of cytochrome *c* release (2). Therefore, it is possible that E1A may alter mitochondrial potential by downregulating Akt and upregulating p38, thereby facilitating the release of cytochrome *c* upon treatment with chemotherapeutic drugs, such as paclitaxel. Thus, downregulation of key survival factor Akt activity and subsequent upregulation of a proapoptotic factor p38 activity by E1A may constitute a fundamental approach for E1A-mediated sensitization to apoptosis.

The mechanisms underlying E1A-mediated downregulation of Akt activity are not yet clear. Obviously, E1A-mediated downregulation of Her-2/neu and/or Axl may contribute to

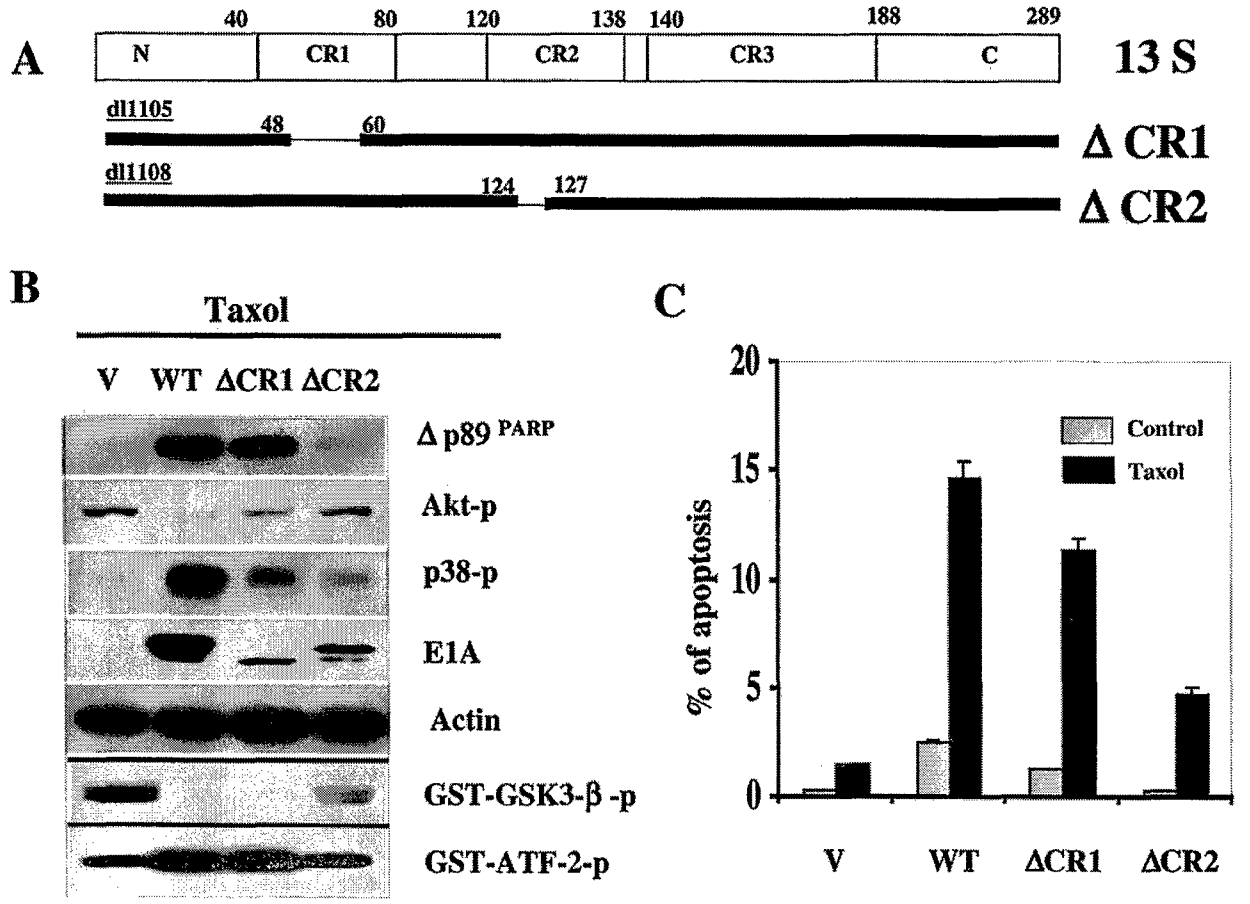


FIG. 7. The CR2 domain of E1A is required for downregulation of Akt and sensitization to drug-induced apoptosis. (A) Domain structure and map for deletion mutation of CR1 and CR2. (B) Akt and p38 kinase activity were measured, and Western blot analysis of PARP cleavage, phospho-Akt, phospho-p38, or E1A expression was performed in different domain deletion mutant stable cells. Actin was used as a loading control. (C) FACS analysis of apoptosis of wild-type (WT) E1A and different domain deletion mutant E1A stable cells with or without treatment with 0.01 μM paclitaxel for 21 h. V, pSV-neo vector-transfected stable cells.

reduced Akt activation, as activation of either Her-2/neu or Axl leads to PI3K-Akt kinase activation and downregulation of Her-2/neu and/or Axl also contributes to E1A-mediated sensitization to apoptosis (21, 29, 30, 58). However, downregulation of Akt activity by E1A may not necessarily depend on E1A-mediated downregulation of Her-2/neu and/or Axl, because both MDA-MB-231 and MCF-7 cells are low Her-2/neu-expressing cells and MCF-7 cells have an undetectable expression level of Axl (35). In addition, deletion mutation of the CR2 domain affects E1A-mediated downregulation of Akt (Fig. 7B), but it has no effect on E1A-mediated transcriptional repression of Her-2/neu (9). Thus, in addition to the Her-2/neu-dependent pathway, a Her-2/neu-independent pathway must exist for E1A to mediate downregulation of Akt activity leading to sensitization to apoptosis. In addition, overexpression of Her-2/neu or activation of Akt also leads to p53 destabilization (50, 59), suggesting that downregulation of Akt activity by E1A may constitute an alternative pathway for stabilization of p53. Like Her-2/neu and p53, Akt also plays a critical role in the regulation of apoptotic cell death and the development of human cancer. Therefore, E1A-mediated downregulation of Akt and upregulation of p38 activities may have general implications for E1A-mediated tumor suppression and sensitization to apoptosis.

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Phosphorylation/Cytoplasmic Localization of $p21^{Cip1/WAF1}$ Is Associated with HER2/*neu* Overexpression and Provides a Novel Combination Predictor for Poor Prognosis in Breast Cancer Patients

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ABSTRACT

Purpose: **The diversity of biological functions makes $p21^{Cip1/WAF1}$ (p21) a controversial marker in predicting the prognosis of breast cancer patients. Recent laboratory studies revealed that the regulation of p21 function could be related to different subcellular localizations of p21 by Akt-induced phosphorylation at threonine 145 in HER2/*neu*-overexpressing breast cancer cells. The purpose of this study was to verify these findings in clinical settings.**

Experimental Design: **The expression status of the key biological markers in the HER2/*neu*-Akt-p21 pathway in 130 breast cancer specimens was evaluated by immunohistochemical staining and correlated with patients' clinical parameters and survival. In addition, an antibody against phospho-p21 at threonine 145 [phospho-p21 (T145)] was also used for better validation of these findings.**

Results: **Cytoplasmic localization of p21 is highly correlated with overexpression of phospho-p21 (T145). Both cytoplasmic p21 and overexpression of phospho-p21 (T145) are associated with high expression of HER2/*neu* and phospho-Akt. Cytoplasmic localization of p21 and overexpression of phospho-p21 (T145), HER2/*neu*, and phospho-Akt are all associated with worse overall survival. Multivariate analysis of the Cox proportional hazard regression model revealed that cytoplasmic p21 and overexpression of HER2/*neu* are independently associated with increased risk of death. Combining these two factors stratified patients' survival into four distinct groups, with a 5-year survival rate of 79% in low HER2/*neu* and negative/nuclear p21 patients, 60% in high HER2/*neu* and negative/nuclear p21 patients, 29% in low HER2/neu and cytoplasmic p21 patients, and 16% in high HER2/*neu* and cytoplasmic p21 patients.**

Conclusions: **The present study, in addition to supporting the mechanisms of p21 regulation derived from laboratory investigation, demonstrates the prognostic importance of phospho-p21 (T145) for the first time and also provides a novel combination of p21 and HER2/*neu* for better stratification of patients' survival than any single clinicopathological or biological marker that may play important diagnostic and therapeutic roles for breast cancer patients.**

INTRODUCTION

Overexpression or amplification of the receptor tyrosine kinase HER2/*neu* (also known as ErbB2) has been noted in ~30% of breast cancer patients and is frequently associated with shorter survival and poor prognostic features, including earlier relapse and increased number of lymph node metastases (1). The underlying mechanisms by which HER2/*neu* promotes tumorigenesis, invasiveness, and metastasis of cancer cells have been extensively studied (2). The phosphatidylinositol-3-OH kinase/Akt pathway is an important HER2/*neu* downstream cascade in preventing cells from undergoing apoptosis and contributing to cell proliferation (3–5). For example, after phosphorylation, the activated Akt can phosphorylate various substrates, such as Bad (6), caspase-9 (7), Forkhead family transcription factors (8, 9), MDM2 (10, 11), and $p21^{Cip1/WAF1}$ (p21; Ref. 12), resulting in either suppression of apoptosis or promotion of cell proliferation. Activation of Akt has also been correlated with poor outcome in breast cancer patients (13).

Among the downstream substrates of Akt, p21 is a critical modulator of cell cycle and cell survival, although its regulation and function have largely remained unclear. p21 was initially considered to be an inhibitor of cell cycle progression and has been shown to suppress tumor formation in xenograft models (14, 15). However, several recent studies have suggested that this protein can also promote cell survival and cell cycle progression (16–18). In addition, elevated p21 protein levels have been observed in various aggressive malignancies, such as glioma and leukemia, and may contribute to chemoresistance (19, 20). The role of p21 in breast cancers has also been controversial in laboratory and clinical studies. It has been shown that HER2/*neu*-overexpressing breast cancer cells can induce chemoresistance through increased expression of p21 (21) and that p21

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overexpression is associated with poor prognosis in breast cancer patients (22). On the other hand, several studies indicated that p21 expression provides no prognostic information for patients with breast cancer (23, 24). The contradictory effects on tumorigenesis, as well as inconsistent reports about clinical outcomes of p21, could be related to the subcellular localization of this special protein because recent studies have revealed that the cell growth-inhibitory activity of p21 is strongly correlated with its nuclear localization. However, p21 can also localize in the cytoplasm, where it plays an important role in protecting cells from apoptosis (16, 18) and was associated with poor prognosis in breast cancer patients (25).

The cellular localization of p21 has been proposed to be critical for the regulation of p21 function (26), and we recently identified the mechanism by which p21 is phosphorylated by Akt at a consensus threonine residue (threonine 145), which results in cytoplasmic localization and suppression of growth-inhibiting activity (12). Because our previous findings clearly demonstrated the regulation of p21 localization and function of the HER2/*neu*-Akt pathway in a laboratory setting, to further address how this signaling pathway is related to survival and other clinical parameters of breast cancer patients, we analyzed the expression status of p21, HER2/*neu*, and phospho-Akt by immunohistochemical (IHC) staining in 130 breast cancer specimens and compared their expression levels and subcellular localization with clinical outcome. In addition, we used a newly developed antibody against phospho-p21 at threonine 145 [phospho-p21 (T145)] to further validate the results.

MATERIALS AND METHODS

Patients and Tumor Specimens. We obtained 130 archived blocks containing formalin-fixed, paraffin-embedded infiltrating breast carcinoma from the Department of Pathology, Shanghai East Breast Disease Hospital, People's Republic of China. All of the patients were women with nonmetastatic disease who had undergone mastectomy and axillary lymph node dissection between 1988 and 1994. After surgical treatment, the patients were offered adjuvant chemotherapy and/or radiotherapy and hormone therapy, depending on the number of lymph node metastases, status of menopause, and estrogen and/or progesterone receptor positivity. The clinicopathological characteristics of the study population, including age, tumor size, lymph node status, tumor grade, and estrogen receptor/progesterone receptor positivity, were obtained from medical records. The estrogen and progesterone receptor status was unavailable for 20 and 19 tumor specimens, respectively. The stage was assessed by the TNM clinical staging system of the American Joint Committee on Cancer (27). Patients were followed 4–72 months, with a median follow-up of 48 months.

Generation of Anti-Phospho-p21 (T145) Antibody. The polyclonal antibody against phosphorylated human p21 protein was generated by immunization of rabbits with a carrier protein, keyhole limpet hemocyanin, in conjunction with a phosphorylated 11-mer peptide [KRRQT-(PO_3)-SMTDFY] at the terminal region of the p21 sequence encompassing the Akt phosphorylation site (threonine 145). Peptides were synthesized for antibody production by SynPeptide, Inc. (Dubin, CA) and were checked under stringent analytical specifications, which included high performance liquid chromatography, mass spectrometry, and UV analysis. The polyclonal antibody was also generated and affinity-purified by SynPeptide, Inc.

Immunoprecipitation and Immunoblotting. 293T cells were transiently transfected by use of SN liposome (28). After transfection for 36 h, 293T cells were washed with PBS and scraped into RIPA-B buffer. After brief sonication, the cell lysates were centrifuged at 14,300 × *g* for 30 min at 4°C to remove insoluble cell debris. The supernatant was preincubated with protein G-agarose (Roche) for 1 h at 4°C. Flag-tagged p21 was immunoprecipitated overnight with anti-Flag (M2) antibody (Sigma) and protein G-agarose. The immunocomplex was washed four times with RIPA-B buffer, dissolved in sampling buffer, and subjected to SDS-PAGE; the proteins were then transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA in Tris-buffered saline and were incubated with anti-phospho-p21 (T145) antibody (1:1000 diluted in Tris-buffered saline-Tween containing 3% BSA) and then with horseradish peroxidase-conjugated antirabbit secondary antibodies. The immunoblots were visualized by use of the ECL kit (Amersham Pharmacia Biotech).

IHC Staining for p21, Phospho-p21 (T145), HER2/*neu*, and Phospho-Akt. The immunoperoxidase staining method was modified from the avidin-biotin complex technique as described previously (29). In brief, slides (5 μm) were deparaffinized. After antigen retrieval, the slides were digested in 0.05% trypsin. The endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide, and the slides were then treated with 10% normal goat or horse serum for 30 min. After overnight incubation with primary antibodies, including

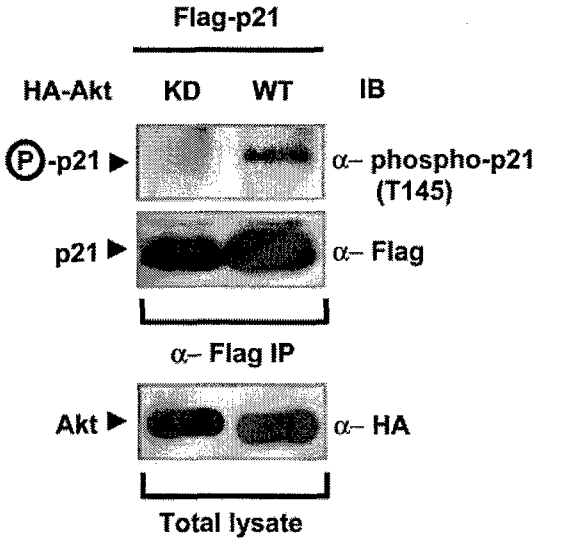

Fig. 1 Detection of p21 (T145) phosphorylation by anti-phospho-p21 (T145) antibody. Flag-tagged p21 and HA-tagged wild-type (*WT*) or kinase-dead Akt (*KD*) were cotransfected transiently into 293T cells. After 36 h of transfection, lysates of cells were subjected to p21 immunoprecipitation (*IP*) with anti-Flag antibody. p21 (T145) phosphorylation by Akt was detected by an anti-phospho-p21 (T145)-specific antibody. Expression levels of Akt (WT) and Akt (KD) were assessed by immunoblotting (*IB*).

(*a*) rabbit polyclonal anti-p21 (c-19; 1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA); (*b*) rabbit polyclonal anti-phospho-p21 (T145) (1:15 dilution; generated by SynPeptide Inc); (*c*) rabbit polyclonal anti-HER2/*neu* (anti-c-erbB-2, 1:300 dilution; DAKO, Carpinteria, CA); and (*d*) rabbit polyclonal anti-phospho-Akt (T308) (1:80 dilution; New England Biolabs Inc.), the slides were incubated with biotinylated secondary antibodies and subsequently incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Antibody detection was performed with the 0.125% aminoethylcarbazole chromogen substrate solution (AEC substrate) from Sigma Chemical Co. After counterstaining with Mayer's hematoxylin (Sigma), the sides were mounted. For negative control, all incubation steps were identical except that PBS was used rather than primary antibody. A previously identified strongly staining tumor tissue section was used as a positive control. The prepared slides were examined by light microscopy.

Fluorescent *in Situ* Hybridization (FISH). The paraffin-embedded tissue sections were baked at 65°C for 2 h and deparaffinized as described previously (29). The FISH assay was performed with the PathVysion HER2 DNA probe Kit (Vysis Inc) according to the manufacturer's recommendation.

Scoring of Immunoreactivity. The immunoreactivity of these antibodies was scored according to the subcellular localization (membrane, nuclear, and/or cytoplasmic), staining intensity (strong, moderate, weak, and faint or slightly above background), and fraction of positive staining. The mean fraction of positive tumor cells was determined in at least nine areas at ×100 or ×200 magnification. p21 immunoreactivity was determined by the percentage of positively stained tumor cells as well as the subcellular localization of staining and was categorized as negative, nuclear, and cytoplasmic. Negative was defined as undetectable cytoplasmic or nuclear staining. Nuclear p21 was defined as the fraction of tumor cells with positive nuclear staining greater than or equal to that of positive cytoplasmic staining. Cytoplasmic p21 was defined as the fraction of cytoplasmic staining greater than that of nuclear staining. HER2/*neu* immunoreactivity was determined by membrane staining and categorized as 0 to 3+. A score of 0 was defined as undetectable staining or membrane staining in <10% of tumor cells, a score of 1+ was defined as faint membrane staining in >10% of tumor cells, a score of 2+ was defined as weak to moderate membrane staining in >10% of tumor cells, and a score of 3+ was defined as intense membrane staining in >10% of tumor cells. The immunoreactivities of phospho-Akt and phospho-p21 (T145) were also ranked into four groups according to the percentage of positively stained tumor cells, including cytoplasmic and nuclear staining: 0, no staining; 1+, <20% cells stained; 2+, 20–49% cells stained; and 3+, >50% cells stained. For analysis, HER2/*neu*, phospho-Akt, and phospho-p21 (T145) expressions was further classified as low (score 0 and 1+) or high (scores 2+ and 3+). The slides were read independently by two investigators without knowledge of the clinical data. If differences between observers occurred, both investigators used a multiheaded microscope to reexamine the slides.

Statistical Analysis. Data on eligible patients were summarized by use of standard descriptive statistics and frequency tabulation. The associations between expression of biomarkers (p21, phospho-p21, HER2/*neu*, and phospho-Akt) and various clinicopathological parameters were assessed by cross-tabulation χ^2 tests. All correlations between each biomarker were analyzed by Kendall's τ-b analysis. The overall survival after surgery was plotted by use of the Kaplan–Meier method. The

Table 1 Clinicopathological and immunohistochemical data for the patients

Characteristic	Patients	
	n	%
Total	130	100
Age (yrs)		
≤48	66	51
>48	64	49
Tumor status		
T_1	66	51
T_2	53	41
T_3	11	8
Lymph node status		
N_0	68	52
N_1	25	19
N_2	32	25
N_3	5	4
Stage		
I	45	35
II	46	35
III	39	30
Tumor grade[a]		
1	28	22
2	43	33
3	59	45
Estrogen receptor		
Negative	68	52
Positive	42	32
Unknown	20	15
Progesterone receptor		
Negative	57	44
Positive	54	41
Unknown	19	15
p21[b]		
Negative	53	41
Nuclear	34	26
Cytoplasmic	43	33
Phospho-p21 (T145)		
Low		
0	64	49
1+	26	20
High		
2+	27	21
3+	13	10
HER2/*neu*		
Low		
0	42	32
1+	42	32
High		
2+	21	16
3+	25	19
Phospho-Akt		
Low		
0	73	56
1+	23	18
High		
2+	14	11
3+	20	15

[a] Tumor grade was classified by WHO criteria (grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated).

[b] Nuclear p21, fraction of nuclear staining greater than or equal to fraction of cytoplasmic staining; cytoplasmic p21, fraction of cytoplasmic staining greater than fraction of nuclear staining.

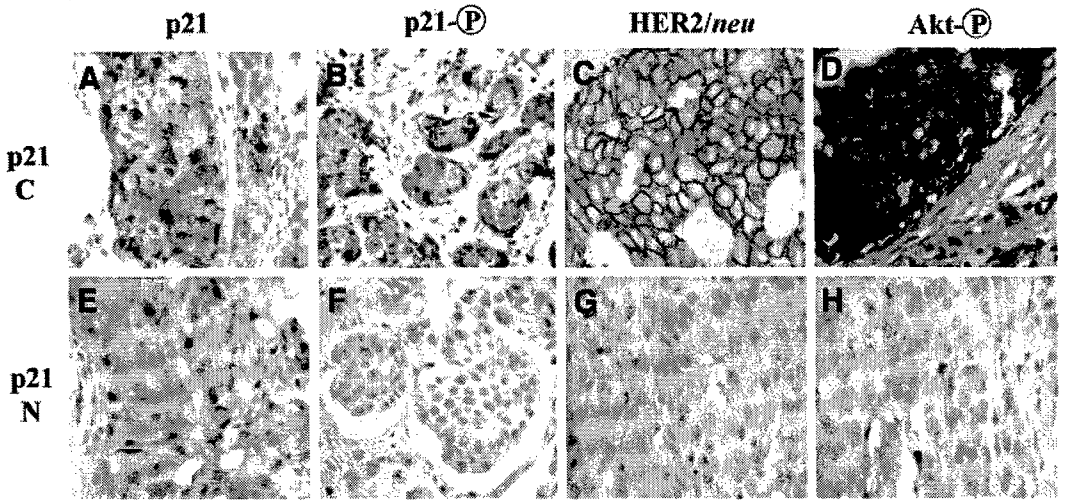

Fig. 2 Representative immunohistochemical pictures of phospho-p21 (T145), HER2/*neu*, and phospho-Akt in cytoplasmic p21 (*C*) or nuclear p21 (*N*) specimens. Tissue sections from patients with cytoplasmic p21 (*A–D*) and nuclear p21 (*E–H*) were stained with specific antibodies against p21 (*A*, cytoplasmic; *E*, nuclear), phospho-p21 (T145) (*B*, overexpression; *F*, negative), HER2/*neu* (*C*, overexpression; *G*, negative), and phsopho-Akt (*D*, overexpression; *H*, negative).

log-rank test was used to analyze differences in survival time. The Cox proportional hazard regression model was used to assess effect of patients' prognostic factors on overall survival. Statistical analysis was performed with SAS 8.0 and S-plus 2000 software. All tests were two-sided, and the level of significance was set at 0.05.

RESULTS

Characterization of Anti-Phospho-p21 (T145) Antibody. Our previous study demonstrated that activated Akt can phosphorylate p21 at threonine 145 and lead to cytoplasmic localization of p21 in HER2/*neu*-overexpressing breast cancer cells (12). To further validate that p21 is phosphorylated on threonine 145 in cancer cells, we developed an anti-phospho-p21 (T145) antibody to recognize this phosphorylation site (see "Materials and Methods"). To characterize the specificity of this antibody, we cotransfected p21 with either wild-type or kinase-dead Akt into 293T cells to determine the specificity of this antibody by a biochemical method. As shown in Fig. 1, phosphorylation of p21 at threonine 145 was detected by this antibody, suggesting that this antibody could recognize phopho-p21 (T145) specifically.

Clinicopathological and IHC Profiles of the Study Population. Patient and tumor characteristics for the entire study population are shown in Table 1. For the 130 patients, the median age was 48 years (range, 26–87 years). No patients had T4 disease or detectable distant metastasis at the time of the surgery. Pathology examination revealed that 118 (91%) of the tumors were infiltrating ductal carcinomas; the remaining 12 (9%) were infiltrating lobular carcinomas. Representative IHC photographs of p21, phospho-p21 (T145), HER2/*neu*, and phospho-Akt are shown in Fig. 2. Positive p21 expression was found in 59% of patients, and the immunoreactivity was in both the cytoplasm and nucleus for the majority of these patients, with 26% mainly in the nucleus and 33% mainly in the cytoplasm. Phospho-p21 (T145) levels were high in 40 (31%) patients, with staining predominantly in the cytoplasm. Phospho-Akt levels were high in 34 (26%) patients, with staining located mainly in the cytoplasm and nucleus. HER2/*neu* levels were high in 46 (35%) patients, with staining mainly in the cell membrane and cytoplasm. To validate the results from the IHC staining, we used FISH to examine randomly selected IHC breast tumors from this cohort. Among HER2/*neu* 3+ and 2+ tissues, we found high degree of correlation, nearly 2+ (87.5%), 3+ (100%), and 0 (100%). Thus, the IHC result is highly consistent with the result from FISH (Table 2 and Fig. 3).

The relationship between various clinicopathological parameters and the biological markers are shown in Table 3. No significant association was noted between the subcellular localization of p21 and age, tumor size, lymph node status, stage, tumor grade, or estrogen and progesterone receptor status. When compared with low expression of phospho-p21, HER2/*neu*, and phospho-Akt, overexpression of these biological markers was also not associated with any clinicopathological parameter of breast cancer patients.

Cytoplasmic p21 Is Highly Correlated with Overexpression of Phospho-p21 (T145), Which Is Located Primarily in the Cytoplasm. To further study the correlation between phospho-p21 (T145) and overall survival of breast cancer patients,

Table 2 Comparison of immunohistochemical staining and fluorescence *in situ* hybridization

	HER2/*neu* IHC			
HER2/*neu* probe[a] FISH[b]	3+	1+	0	Total
Amplified	5	7	0	12
Nonamplified	0	1	5	6
Total	5	8	5	18

[a] Pearson's χ^2 test (SPSS): $P = 0.95$ ($P > 0.05$).
[b] FISH, fluorescence *in situ* hybridization; IHC, immunohistochemical staining.

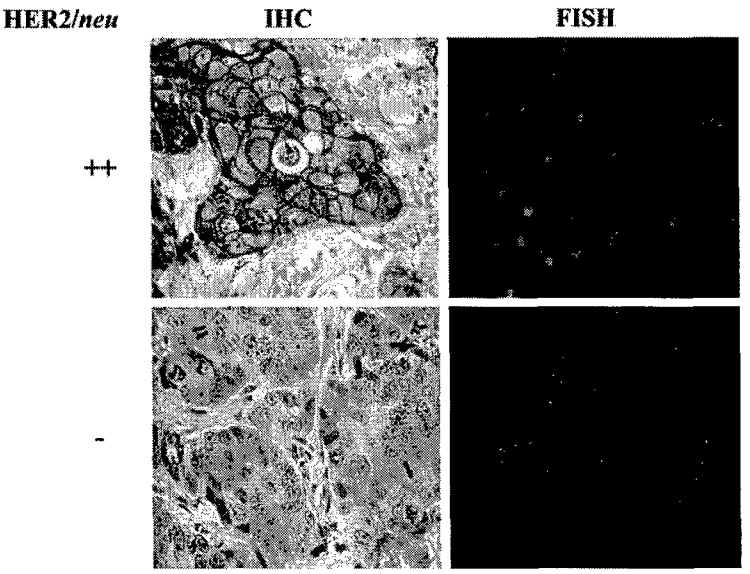

Fig. 3 HER2/*neu* expression and amplification detected by immunohistochemical staining (*IHC*) and fluorescence *in situ* hybridization (*FISH*), respectively. IHC negative (−) is related to FISH nonamplified (−); IHC high positive (++) is correlated to FISH amplified (++). For the FISH assay, *green* is chromosome 17 centromere; and *red* is HER2/*neu*.

we examined surgical specimens from breast cancer patients immunohistochemically, using this antibody. The subcellular location of positive staining was predominantly in the cytoplasm (Fig. 2*B*). Nuclear staining could be detected in only 2 of the 66 specimens with cytoplasmic staining and was very weak. In addition, the expression of phospho-p21 (T145) was highly correlated with the subcellular localization of p21 (Table 4). When p21 was localized mainly in the cytoplasm, phospho-p21 (T145) was likely to be highly expressed (see also Fig. 2, *A* and *B*). In contrast, when p21 was located mainly in the nucleus, phospho-p21 (T145) expression tended to be low or negative [see Fig. 2, *E* and *F*; correlation coefficient (r) = 0.33; P = 0.001].

Table 3 Association of p21, phospho-p21, HER2/*neu*, and phospho-Akt with clinicopathological parameters in 130 breast cancer patients
$P > 0.05$ for all associations between biomarkers and clinicopathological variables analyzed by cross-tabulation (χ^2 test).

	No. of patients (%)			
Parameters	Cytoplasmic p21[a]	High phospho-p21 (2+/3+)	High HER2/*neu* (2+/3+)	High phospho-Akt (2+/3+)
Total (*n*)	43	40	46	34
Age (yrs)				
≤48	20 (15.4%)	18 (13.8%)	25 (19.2%)	16 (12.3%)
>48	23 (17.7%)	22 (16.9%)	21 (16.2%)	18 (13.8%)
Tumor size				
T_1	17 (13.1%)	17 (13.1%)	19 (14.6%)	18 (13.8%)
T_2 and T_3	26 (20.0%)	23 (17.7%)	27 (20.8%)	16 (12.3%)
Lymph node				
Negative	18 (13.8%)	17 (13.1%)	21 (16.2%)	16 (12.3%)
Positive	25 (19.2%)	23 (17.7%)	25 (19.2%)	18 (13.8%)
Stage				
I	9 (6.9%)	12 (9.2%)	14 (10.8%)	14 (10.8%)
II	17 (13.1%)	12 (9.2%)	13 (10.0%)	6 (4.6%)
III	17 (13.1%)	16 (12.3%)	19 (14.6%)	14 (10.8%)
Tumor grade				
1	6 (4.6%)	7 (5.4%)	6 (4.6%)	6 (4.6%)
2	12 (9.2%)	13 (10.0%)	14 (10.8%)	13 (10.0%)
3	25 (19.2%)	20 (15.4%)	26 (20.0%)	15 (11.5%)
Estrogen receptor[b]				
Negative	16 (14.5%)	18 (16.4%)	19 (17.3%)	20 (18.2%)
Positive	15 (13.6%)	15 (13.6%)	17 (15.5%)	9 (8.2%)
Progesterone receptor[c]				
Negative	15 (13.5%)	20 (18.2%)	15 (13.5%)	13 (11.7%)
Positive	16 (14.4%)	9 (8.2%)	21 (18.9%)	16 (14.4%)

[a] p21 status was divided into three groups: negative, nuclear, and cytoplasmic.
[b] Data from 20 patients are missing.
[c] Data from 19 patients are missing.

Table 4 Correlation between subcellular localization of p21 and immunoreactivity of phospho-p21 (T145)
$r = 0.33$; $P = 0.001$ (Kendall's τ -b analysis).

Phospho-p21 staining	p21 subcellular localization, *n* (%)		
	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total[a] ($n = 77$)
0	16 (21%)	6 (8%)	22 (29%)
1+	8 (10%)	10 (13%)	18 (23%)
2+	6 (8%)	18 (23%)	24 (31%)
3+	4 (5%)	9 (12%)	13 (17%)

[a] Fifty-three patients with negative staining for p21 were excluded.

Table 5 Correlation between immunoreactivity of HER2/*neu* and phospho-Akt
$r = 0.45$; $P < 0.0001$.

Phospho-Akt staining	HER2/*neu* staining, *n* (%)				
	0 ($n = 42$)	1+ ($n = 42$)	2+ ($n = 21$)	3+ ($n = 25$)	Total ($n = 130$)
0	38 (29%)	20 (15%)	8 (6%)	7 (5%)	73 (56%)
1+	3 (2%)	10 (8%)	6 (4%)	4 (3%)	23 (18%)
2+	1 (1%)	7 (5%)	2 (2%)	4 (3%)	14 (11%)
3+	0 (0%)	5 (4%)	5 (4%)	10 (8%)	20 (15%)

Cytoplasmic Localization of p21 and Overexpression of Phospho-p21 (T145) Are Associated with Overexpression of HER2/*neu* and Phospho-Akt. Because we previously demonstrated that activated Akt phosphorylates p21 and determined its subcellular localization in HER2/*neu*-overexpressing cells (12), we examined the correlations among these molecules in this pathway. As shown in Table 5, the level of phospho-Akt expression was strongly correlated with the level of HER/*neu* expression, supporting the previous report that HER2/*neu* overexpression activates Akt by phosphorylation of Akt (12). The association between the levels of HER2/*neu* or phospho-Akt expression and the cellular localization of p21 was also significant (Tables 6 and 7), indicating that when HER2/*neu* or phospho-Akt was highly expressed, p21 was localized mainly in the cytoplasm (see Fig. 2, *A*, *C*, and *D*). In contrast, when the levels of HER2/*neu* or phospho-Akt expression were low or negative, p21 tended to be negative or localized mainly in the nucleus (see Fig. 2, *E*, *G*, and *H*). Furthermore, the levels of HER2/*neu* and phospho-Akt expression were positively associated with the levels of phospho-p21 (T145) expression ($r = 0.49$, $P < 0.001$ for HER2/*neu*; $r = 0.52$, $P < 0.001$ for phospho-Akt). All of these data consistently support the hypothesis that overexpression of HER2/*neu* can activate/phosphorylate Akt, which in turn phosphorylates p21 at threonine 145 and leads to cytoplasmic localization of p21.

Cytoplasmic Localization of p21 and Overexpression of Phospho-p21 (T145) Are Associated with Poor Patient Survival. We next sought to clinically determine whether subcellular localization of p21 and the levels of phospho-p21 (T145)

Table 6 Correlation between subcellular localization of p21 and HER2/*neu* immunoreactivity
$r = 0.37$; $P = 0.0003$.

HER2/*neu* staining	p21 subcellular localization, *n* (%)			
	Negative ($n = 53$)	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total ($n = 130$)
0	28 (22%)	10 (8%)	4 (3%)	42 (32%)
1+	15 (12%)	13 (10%)	14 (11%)	42 (32%)
2+	5 (4%)	5 (4%)	11 (9%)	21 (16%)
3+	5 (4%)	6 (5%)	14 (11%)	25 (19%)

Table 7 Correlation between subcellular localization of p21 and immunoreactivity of phospho-Akt
$r = 0.32$; $P = 0.0011$.

Phospho-Akt staining	p21 subcellular localization, *n* (%)			
	Negative ($n = 53$)	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total ($n = 130$)
0	38 (29%)	21 (16%)	14 (11%)	73 (56%)
1+	10 (8%)	3 (2%)	10 (8%)	23 (18%)
2+	4 (3%)	3 (2%)	7 (5%)	14 (11%)
3+	1 (1%)	7 (5%)	12 (9%)	20 (15%)

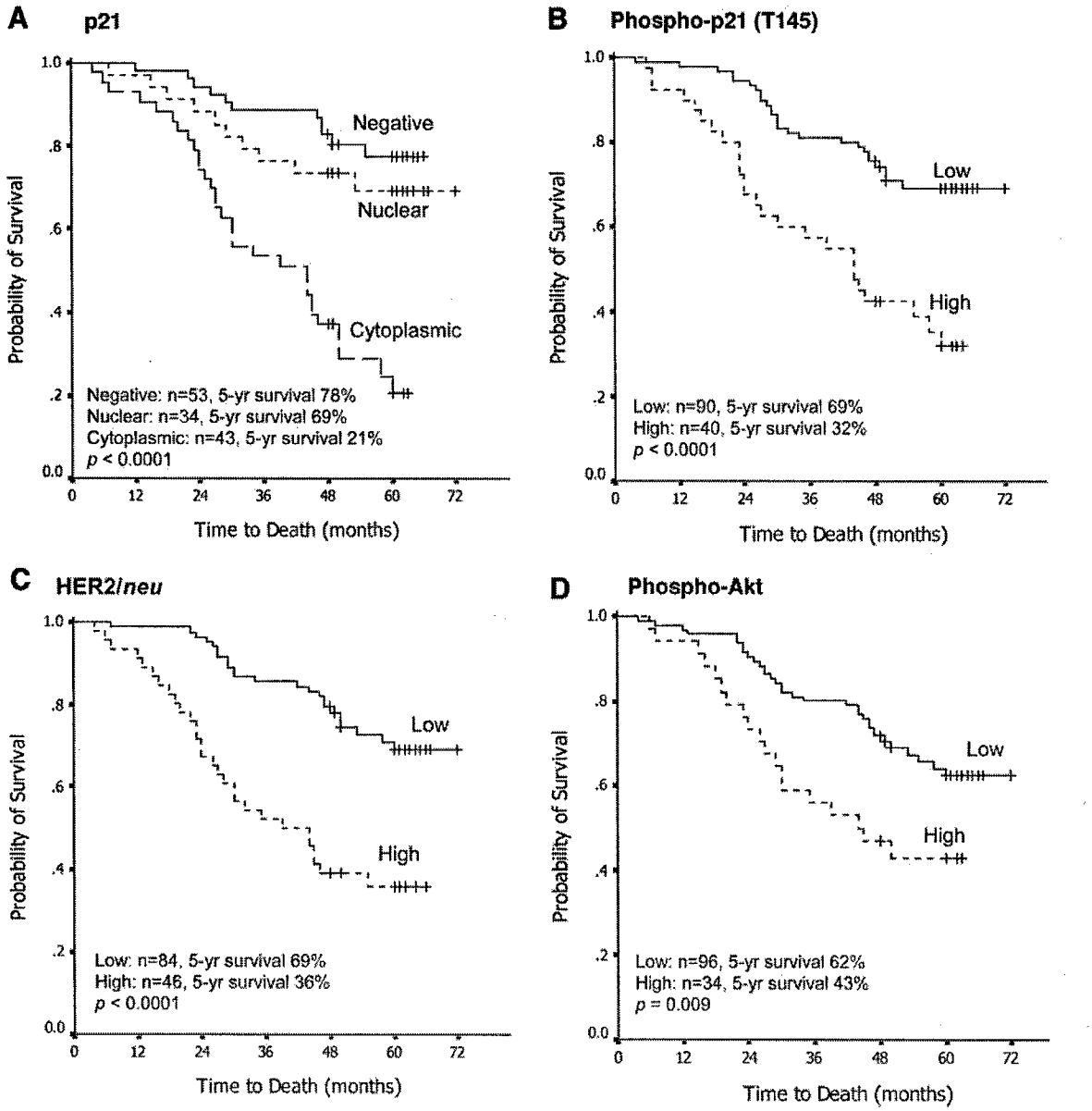

Fig. 4 Kaplan-Meier analyses of overall survival of breast cancer patients. Survival curves are stratified by expression status of p21 (*A*), phospho-p21 (T145) (*B*), HER2/*neu* (*C*), and phospho-Akt (*D*). Differences in survival distributions were evaluated by log-rank tests.

expression affected patient survival. We found that the overall survival of patients with p21 localized in the cytoplasm was much worse than that of patients in whom p21 was negative or localized in the nucleus ($P < 0.0001$; Fig. 4*A*). However, the difference in overall survival between patients negative for p21 and for nuclear p21 was not significant ($P = 0.32$). Consistent with cytoplasmic localization of p21, high levels of phospho-p21 (T145) were also associated with poor patient survival compared with low phospho-p21 (T145) levels (Fig. 4*B*). In addition, poor overall survival was noted in patients with overexpression of HER2/*neu* and phospho-Akt (Fig. 4, *C* and *D*). The effects of p21 subcellular localization and phospho-p21 (T145), HER2/*neu*, and phospho-Akt status on disease-free survival revealed trends similar to those for overall survival (data not shown).

Combined Subcellular Localization of p21 and Expression Status of HER2/*neu* Provide a Better Prognostic Factor. To determine the prognostic importance of clinicopathological parameters and the HER2/*neu*-Akt-p21 pathway members in breast cancer, we performed Cox proportional hazard regression analyses on 130 breast cancer patients. Univariate analysis revealed that large tumor size; advanced nodal status; advanced TNM stage; overexpression of HER2/*neu*, phospho-Akt, and phospho-p21 (T145); and cytoplasmic localization of p21 were associated with a poor outcome (Table 8*A*). In multivariate analysis, only large tumor size, advanced nodal status, overex-

Table 8 Cox's proportional hazard model analysis of prognostic factors in breast cancer patients

Variable	Parameter estimate	SE	*P*	Relative risk
A. Univariate analysis				
Age (> 48 *vs.* ≤48 yrs)	0.41	0.28	0.14	1.50
Tumor (II *vs.* I)	0.91	0.32	0.004	2.49
Tumor (III *vs.* I)	1.95	0.42	<0.0001	7.03
Node (N_1 *vs.* N_0)	0.68	0.40	0.09	1.97
Node (N_2 or N_3 *vs.* N_0)	0.80	0.37	0.033	2.22
Stage (II or III *vs.* I)	1.17	0.37	0.001	3.23
Grade (2 or 3 *vs.* 1)	0.69	0.41	0.09	1.98
HER2/*neu* (high *vs.* low)	1.31	0.28	<0.0001	3.72
Phospho-Akt (high *vs.* low)	0.77	0.29	0.008	2.16
Phospho-p21 (T145) (high *vs.* low)	1.22	0.28	<0.0001	3.39
p21 (cytoplasmic *vs.* others)	1.55	0.29	<0.0001	4.73
HER2/*neu* (low) + p21 (cytoplasmic)[a]	1.35	0.43	0.0018	3.85
HER2/*neu* (high) + p21 (neg.[b]/nuclear)[a]	1.04	0.44	0.018	2.83
HER2/*neu* (high) + p21 (cytoplasmic)[a]	2.32	0.37	<0.0001	10.18
B. Multivariate analysis				
Tumor status (T_2 *vs.* T_1)	0.42	0.33	0.21	1.52
Tumor status (T_3 *vs.* T_1)	2.17	0.47	<0.0001	8.77
Node (N_1 *vs.* N_0)	0.56	0.41	0.17	1.75
Node (N_2 or N_3 *vs.* N_0)	0.70	0.39	0.071	2.01
HER2/*neu* (high *vs.* low)	1.09	0.31	0.001	2.97
p21 (cytoplasmic *vs.* others)	1.31	0.31	<0.0001	3.71
HER2/*neu* (low) + p21 (cytoplasmic)[a]	1.37	0.44	0.002	3.92
HER2/*neu* (high) + p21 (neg./nuclear)[a]	1.15	0.45	0.011	3.15
	2.41	0.39	<0.0001	11.08

[a] The combination of HER2/*neu* (low) and p21 (negative/nuclear) is used as the baseline. The relative risk of the combined HER2/*neu* (low) and p21 (cytoplasmic) against baseline is 3.85 by univariate analysis, 3.92 by multivariate analysis.
[b] neg., negative.

pression of HER2/*neu*, and cytoplasmic localization of p21 were significant and independent prognostic factors in this study (Table 8*B*). To better predict the prognosis of breast cancer patients, we combined the expression status of HER2/*neu* and subcellular localization of p21, creating a new prognostic factor. To simplify the classification, patients negative for p21 and nuclear p21 were combined into one group (negative/nuclear p21) because their survival rates were not significantly different. When patients' overall survival was stratified by this new combined factor, the 5-year survival rates were, respectively, 79, 60, 29, and 16% for the four groups: (*a*) patients with low HER2/*neu* and negative/nuclear p21; (*b*) patients with high HER2/*neu* and negative/nuclear p21; (*c*) patients with low HER2/*neu* and cytoplasmic p21; and (*d*) patients with high HER2/*neu* and cytoplasmic p21 ($P < 0.001$; Fig. 5*A*). This new factor was found to be a more powerful predictor of patient survival than any individual clinicopathological or biological marker in this study by Cox regression model and Kaplan-Meier survival analyses (see also Table 8 and Fig. 4), with a relative risk by multivariate analysis of 11.08 ($P < 0.0001$) for patients with overexpressed HER2/*neu* and cytoplasmic p21. Furthermore, the predictive power and accuracy of this new prognostic factor could be comparable to TNM staging, the "gold standard" prognostic determinant of breast cancer (Fig. 5*B*).

DISCUSSION

The present study examined the expression status of the key members of the HER2/*neu*-Akt-p21 pathway in breast cancers as well as their relationship with clinicopathological parameters and patient survival. We demonstrated that the cytoplasmic localization of p21 is highly correlated with overexpression of phospho-p21 (T145). Both cytoplasmic p21 and overexpression of phospho-p21 (T145) are associated with high expression levels of HER2/*neu* and phospho-Akt, and all of these biological conditions are associated with poor survival of breast cancer patients. In addition, by the multivariate Cox proportional hazard model, overexpression of HER2/*neu* and cytoplasmic p21 were found to be significant and independent factors in predicting outcome. Further stratification of patients' survival by combined HER2/*neu* and p21 status provides a more accurate prediction than any individual clinicopathological and biological factor. The predictive power of this new combined factor could be comparable to that of TNM staging.

The role of p21 in the prognosis of breast cancer patients has been controversial. Several studies demonstrated that by using a monoclonal antibody (clone 4D10, mouse IgG1 subtype; Novocastra, Newcastle upon Tyne, United Kingdom), the expression of p21 was predominantly in the nucleus and provided no prognostic information (23, 24). However, using another monoclonal antibody (Ab-1; Calbiochem, Cambridge, MA), one study revealed that p21 immunoreactivity was both cytoplasmic and nuclear in the majority of breast cancers and that cytoplasmic p21, rather than the total p21 level, was associated with poor survival (25). In our study, two antibodies that recognized different forms of p21 were used. With anti-p21 (c-19; Santa Cruz Biotechnology), the immunoreactivity was both cytoplas-

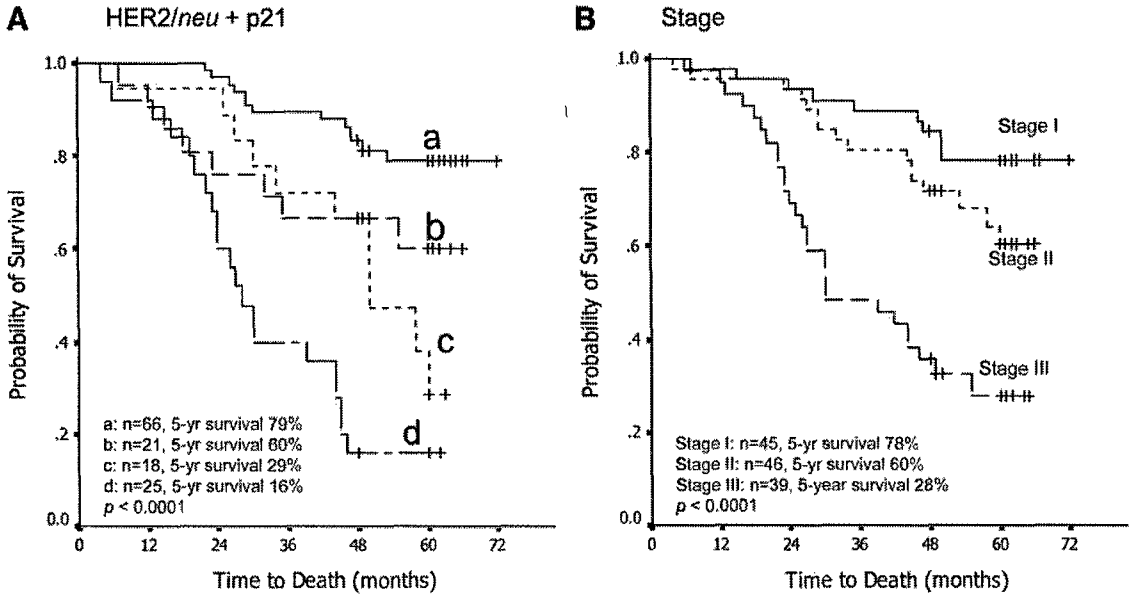

Fig. 5 Kaplan-Meier analyses of overall survival of breast cancer patients. Survival curves are stratified by a combination of expression status of HER2/*neu* and subcellular localization of p21 (*A*; *line a*, low HER2/*neu* and negative/nuclear p21; *line b*, high HER2/*neu* and negative/nuclear p21; *line c*, low HER2/*neu* and cytoplasmic p21; *line d*, high HER2/*neu* and cytoplasmic p21) and TNM stage (*B*). Differences in survival distributions were evaluated by log-rank tests.

mic and nuclear. With anti-phospho-p21 (T145), the immunoreactivity was primarily in the cytoplasm. Both antibodies consistently showed that p21, particularly the phosphorylated form, can be localized in the cytoplasm and that cytoplasmic localization of p21 was associated with a poor outcome for breast cancer patients. On the other hand, nuclear p21 was more closely associated with low levels of HER2/*neu* and phospho-Akt, and the overall survival of patients with nuclear p21 was not significantly different from that of those negative for p21. Our results suggest that the previous controversy over clinical results might be caused by the different antibodies used in IHC staining and different interpretation of subcellular localization of p21.

Many laboratory findings suggest that p21 might play dual roles in regulating cell cycle progression and apoptosis by changing its subcellular localization. Our data demonstrate that cytoplasmic p21 has a prognostic implication different from that of nuclear p21 in breast cancer patients. However, the molecular mechanisms by which p21 changes its subcellular localization remained elusive until we reported that Akt may play a pivotal role in p21 localization (12). In that study, we showed that activated Akt physically associates with and phosphorylates p21 at threonine 145, resulting in cytoplasmic localization of p21. Blocking of the Akt pathway with an Akt (KD) mutant restores the nuclear localization and cell-growth inhibition of p21. In the present study, we consolidate these findings in a clinical setting by showing that overexpression of activated/phosphorylated Akt is highly correlated with overexpression of phospho-p21 (T145) and cytoplasmic localization of p21 and that phospho-p21 (T145), when detected, is primarily localized in the cytoplasm. Our results also indicate that the Akt-p21 pathway plays an important role in the prognosis of breast cancer patients because high levels of phospho-Akt and phospho-p21 (T145) and cytoplasmic localization of p21 are associated with poor patient survival.

Although we clearly showed that activated Akt induces cytoplasmic localization of p21 and correlates with shorter survival of patients, the clinical sequence by which cytoplasmic p21 leads to poor prognosis remains unclear. Using a multivariate Cox proportional hazard model and analyses of the correlation with clinicopathological parameters, we found that cytoplasmic p21 is an independent prognostic factor that correlated with no clinicopathological parameters, such as large tumor size, advanced lymph node metastases, or poor tumor grade in this study. However, recent studies revealed that breast cancer cells and leukemia cells can induce resistance to chemotherapeutic drugs through overexpression of p21 (20, 21) and that activation of Akt can lead to cytoplasmic localization and stabilization of p21 (12, 30). We therefore hypothesize that after phosphorylation by activated Akt, p21 can increase its expression levels, translocate into the cytoplasm, induce the phenotype of chemoresistance, and finally lead to poor survival of breast cancer patients irrespective of tumor size, grade, and lymph node status.

The relationship between HER2/*neu* and the Akt-p21 pathway has rarely been addressed. In this study, we found that cytoplasmic localization of p21 and overexpression of phospho-p21 (T145) and phospho-Akt were highly associated with overexpression of HER2/*neu*, in concordance with our previous finding that the Akt pathway is required for HER2/*neu*-mediated cell proliferation (12). However, our patients' data also implied that overexpression of HER2/*neu* may not be the only mechanism to induce Akt activation and p21 phosphorylation/cytoplasmic localization in breast cancer patients because the mul-

tivariate Cox proportional hazard model revealed that HER2/*neu* overexpression and p21 cytoplasmic localization are significant but independent prognostic factors, suggesting that, in addition to HER2/*neu*, other pathways could activate Akt or phosphorylate p21 and cause p21 to localize in the cytoplasm. Furthermore, overexpression of HER2/*neu* can reduce patient survival through many pathways other than Akt-p21.

The advance of laboratory studies has provided many biological markers to predict the prognosis of patients and provide targets for therapy. The accumulated results of studies also indicate that, with appropriate selection, multiple markers might be more informative than any single marker for the prediction of clinical outcome in breast cancer patients (31). In this study, in addition to consolidating the mechanisms of p21 regulation through the activation of the HER2/Akt pathway, we also demonstrated that cytoplasmic p21 was an independent prognostic factor in breast cancer patients. A novel combination of subcellular localization of p21 and expression status of HER2/*neu* clearly stratified the patients into four distinctly different survival groups. Among them, the 5-year survival rate of patients with low HER2/*neu* and negative/nuclear p21 was 79%, in contrast to only 16% in those patients with high HER2/*neu* and cytoplasmic p21. This novel combination not only provides a better prognostic prediction than any individual clinicopathological or biological marker, it also indicates that targeting only one molecule, such as HER2/*neu,* could be insufficient. Novel therapeutic agents that target phosphorylation/cytoplasmic localization of p21 may also contribute to optimal treatment of breast cancer patients.

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Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by a nonviral-mediated systemic delivery of E1A gene in orthotopic xenograft human breast cancer

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Paclitaxel (Taxol) is a promising frontline chemotherapeutic agent for the treatment of human breast and ovarian cancers. The adenoviral type 5 E1A gene has been tested in multiple clinical trials for its anticancer activity. E1A has also been shown to sensitize paclitaxel-induced killing in E1A-expressing cells. Here, we show that E1A can sensitize paclitaxel-induced apoptosis in breast cancer cells in a gene therapy setting by an orthotopic mammary tumor model. We first showed that expression of E1A enhanced *in vitro* paclitaxel cytotoxicity, as compared to the control cells. We then compared the therapeutic efficacy of paclitaxel between orthotopic tumor models established with vector-transfected MDA-MB-231 (231-Vect) *versus* 231-E1A stable cells, using tumor weight and apoptotic index (TUNEL assay) as the parameters. We found paclitaxel was more effective in shrinking tumors and inducing apoptosis in tumor models established with stable 231-E1A cells than the control 231-Vect cells. We also tested whether E1A could directly enhance paclitaxel-induced killing in nude mice, by using a nonviral, surface-protected cationic liposome to deliver E1A gene via the mouse tail vein. We compared the therapeutic effects of E1A gene therapy with or without Taxol chemotherapy in the established orthotopic tumor model of animals inoculated with MDA-MB-231 cells, and found that a combination of systemic E1A gene therapy and paclitaxel chemotherapy significantly enhanced the therapeutic efficacy and dramatically repressed tumor growth ($P<.01$). In addition, survival rates were significantly higher in animals treated with combination therapy than in the therapeutic control groups (both $P<.0001$). Thus, the E1A gene therapy indeed enhances the sensitivity of tumor cells to chemotherapy in a gene therapy setting and, the current study provides preclinical data to support combination therapy between E1A gene and chemotherapy for future clinical trials.

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Amplification and overexpression of *Her-2/neu* (or c-erbB-2) oncogene have been found in approximately 25% of human breast carcinomas and associated with poor prognosis.[1] Using *Her-2/neu* as a target for the development of therapeutic agents proves to be an effective approach. For example, Herceptin, a humanized monoclonal antibody against *Her-2/neu*, has demonstrated its efficacy by either Herceptin alone or in combination with cisplastin as a first-line therapy for the treatment of breast cancer patients with advanced disease whose tumors overexpressed *Her-2/neu*.[2,3] We have previously reported that the adenoviral type-5 E1A gene product also repressed *Her-2/neu* oncogene overexpression and suppressed the tumorigenic and metastatic potential of *Her-2/neu*-transformed cells.[4]

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The adenoviral type-5 and type-2 E1A were reported originally as oncogenes, which could cooperate with other viral and cellular oncogenes to transform primary culture cells but not established cell lines, distinct from the type-12 E1A, a potent oncogene that can transform established cell lines.[5] However, E1A has not been associated with human malignancies despite extensive studies trying to identify such a link. Instead, E1A was shown to suppress experimental metastasis of rodent cells transformed by the *ras* oncogene[6–8] and the *Her-2/neu* oncogene[9] and metastasis of certain human cancer cell lines.[10] In addition, increasing experimental data indicate that E1A was able to inhibit the tumorigenicity of the transformed rodent cells as well as of the human cancer cell lines.[5,10] Therefore, based on its ability to suppress both tumorigenicity and metastasis, E1A has been considered as a tumor suppressor gene[5,11–13] and translated into multiple clinical trials.[14–16]

In addition to the tumor suppressor activities, expression of the E1A gene in stably transfected normal fibroblast and human cancer cells has also been shown

to increase sensitivity to the *in vitro* cytotoxicity of several anticancer drugs, such as etoposide and cisplatin in normal fibroblasts and sarcoma cells, Adriamycin in colon and hepatocellular carcinoma cells, Gemcitabine in hepatocellular and breast cancer cells, and paclitaxel in breast and ovarian cancers.[17–27] Our previous studies demonstrated that E1A can sensitize paclitaxel-induced cell killing in *Her-2/neu*-overexpressing cancer cells.[22,23] We did not observe E1A-induced paclitaxel sensitization for *Her-2/neu* low-expressing cells under the same condition, in which the paclitaxel concentration is too high to observe sensitization effect in the *Her-2/neu* low-expressing cells (please see Discussion later).[22] However, in our recent study, we found that expression of E1A in *Her-2/neu* low-expressing breast cancer MDA-MB-231 and MCF-7 cells sensitized cells to different categories of anticancer drug-induced apoptosis at a varying extent, these drugs include paclitaxel, cisplatin, Adriamycin, Gemcitabine, and methotrexate.[26] Since these drugs exert there anticancer activity through different modes of actions, we showed that E1A-mediated sensitization to different types of anticancer drug-induced apoptosis was achieved through a general cellular pathway that regulate the cell survival and cell death signalings.[26] In the current study, we further examine the E1A-induced chemosensitization for the *Her-2/neu* low-expressing cancer cells in a mammary tumor model using the newly developed systemic nonviral delivery system SN, which contains a surface-protection polymer to stabilize the liposome-DNA particles for intravenous (i.v.) injection.[28–30] We tested if combination of E1A gene therapy with paclitaxel chemotherapy will enhance the cytotoxicity and antitumor effect of paclitaxel in an orthotopic breast cancer xenograft established by inoculation of low *Her-2/neu*-expressing MDA-MB-231 cells in nude mice. We found that combination of systemic E1A gene therapy with paclitaxel significantly enhanced paclitaxel-induced apoptosis and prolonged animal survival rates in the orthotopic model *in vivo*. Thus, the current study provides further experimental support for combination therapy between E1A gene therapy and paclitaxel chemotherapy.

Materials and methods

Cell lines

Human breast cancer MDA-MB-231 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium/F-12 (Life Technologies Inc., Rockville, MD) supplemented with 10% fetal bovine serum. The stable E1A-expressing cell lines were established as previously described.[31]

Paclitaxel

A stock solution of paclitaxel (Taxol, Bristol-Myers Squibb Co., Princeton, NJ) was stored at −80°C before use. For *in vitro* use, paclitaxel was diluted in serum-free medium at the required concentration. For *in vivo* use, paclitaxel was diluted in normal saline (NS) to a dose of 10 mg/kg in 150 μl per injection, once a week for 6 weeks.

Formulation

The gene-delivery system, termed SN, was described by Zou *et al*.[28,29] The DNA was entrapped in the SN-liposome after the thin-lipid film was hydrated and extruded through a filter with 0.2-μm-diameter pores (Gelman Sciences; Ann Arbor, MI).

MTT assay

The metabolic conversion of tetrazolium salt (MTT) to formazan was used to indirectly measure the number of viable cells after exposure to Taxol. Cells (3×10^3/well) were seeded in triplicate in 96-well culture plates in 0.2 ml of culture medium and allowed to adhere for 24 hours. After exposure to Taxol for the indicated time periods, 20 μl of MTT was added to each well. Cells were cultured for an additional 2 hours, and 100 μl of extraction buffer (20% SDS in 50% *N,N*-dimethyl formamide, pH 4.7) was added to the culture medium. The cells were incubated overnight at 37°C, and the plate absorbency was measured at 570 nm.

Propidium iodide staining and FACS analysis

Samples of 2×10^6 cells were collected, washed once with phosphate-buffered saline (PBS), and fixed with 70% ice-cold ethanol overnight. After fixation, cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing 50 μg/ml of propidium iodide (Sigma, St Louis, WA). The staining was performed at 4°C for at least 30 minutes, and samples were analyzed using a FACScan (Becton-Dickinson, San Jose, CA) in the core facility at The University of Texas M.D. Anderson Cancer Center.

Athymic nude mice

Female athymic BALB/c-*nu/nu* mice, 4–6 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). The animals were allowed to acclimate for 7 days before the study initiation. All of the animals were housed under pathogen-free conditions, and were given water and chow *ad libitum*. Animal care and use were in accordance with Institutional and NIH guidelines.

Establishment of orthotopic breast cancer model and systematic E1A gene therapy in nude mice

MDA-MB-231 cells (1×10^6 cells/0.1 ml) or 231-E1A cells (2×10^6 cells/0.1 ml) were subcutaneously injected into the mammary fat pad (m.f.p.) of female, athymic mice. Tumors were allowed to develop for 21 days and mice were then randomly grouped and treated with SN-liposome alone (SN, i.v.), Taxol alone (i.p., 10 mg/kg/injection, once a week for 6 weeks), E1A alone (i.v., 15 μg/mouse/injection, injection into mouse tail vein twice a week for 6 weeks), or E1A (i.v., 15μg/mouse/injection) plus Taxol (i.p., 10 mg/kg/injection, 24 hours after E1A

injection, for 6 weeks). Both maximum and minimum diameters of the resulting tumors were measured twice a week using a slide caliper. Tumor volumes were calculated by assuming a spherical shape and using the formula, volume $= 4/3r^3$, where $r = 1/2$ of the mean tumor diameter measured in two dimensions. The mice were killed when their tumors were larger than 2 cm in diameter; otherwise, they would keep growing and would remain as subjects for survival rate analysis.

Immunohistochemical analysis

To evaluate expression of E1A in stable MDA-MB-231 cells, cells in exponential growing were harvested and washed with PBS twice and then cytospuned. Cytospun cells were fixed for 10 minutes in 4% formaldehyde (Sigma), washed with PBS, dried, and stored at −80°C or subject to immunohistochemical analysis. To evaluate expression of E1A protein level in tumor tissues *in vivo*, stable E1A-expressing cells or control vector cells were inoculated into m.p.f. of nude mice. After a palpable tumor was formed, in approximately 4–6 weeks, the tumor was dissected and fixed in 10% formalin and embedded in paraffin blocks. Paraffin-embedded sections were then pretreated with dewax and rehydrated. They were washed in xylene and rehydrated through a graded series of ethanol and redistilled water. The paraffin sections or cytospun slides were incubated with E1A M73 monoclonal antibody (Oncogene Science, Inc., Cambridge, MA) diluted 1:20. The slides were then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) or biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:200 in PBS. The slides were then incubated with an avidin–biotin–peroxidase complex (Vector Laboratories), and the peroxidase-catalyzed product was visualized with 0.125% aminoethyl carbazole chromogen buffer (Sigma Chemical Co.).

In vivo apoptotic (TUNEL) assay

For *in vivo* apoptotic assay, tumors were fixed in 10% formalin and embedded in paraffin blocks as described above. Tissue sections were incubated with proteinase K (20 μg/ml in 10 mM Tris/HCl, pH 7.4–8.0, for 15 minutes at 37°C), permeabilized in 0.1% Triton-X-100 in 0.1% sodium citrate, and then labeled with the TUNEL (deoxynucleotide transferase-mediated dUTP-biotin nick end labeling) reaction mixture (Boehringer Mannheim, Indianopolis, IN), according to the manufacturer's protocol. Briefly, biotinylated nucleotide mix and TdT enzyme were added and incubated for 1 hour at 37°C; slides were washed in PBS, blocked in hydrogen peroxide, and incubated in streptavidin horseradish peroxidase. The slides were developed in 3,3′-diaminobenzidine and counterstained with hematoxylin. The apoptotic cells (brown staining) were counted under a microscope. The apoptosis index was defined by the percentage of brown cells among the total cells of each sample. In total, 10 fields with >200 cells in each were randomly counted for each sample.

Results

E1A-mediated sensitization to paclitaxel correlated with E1A-induced apoptosis in vitro

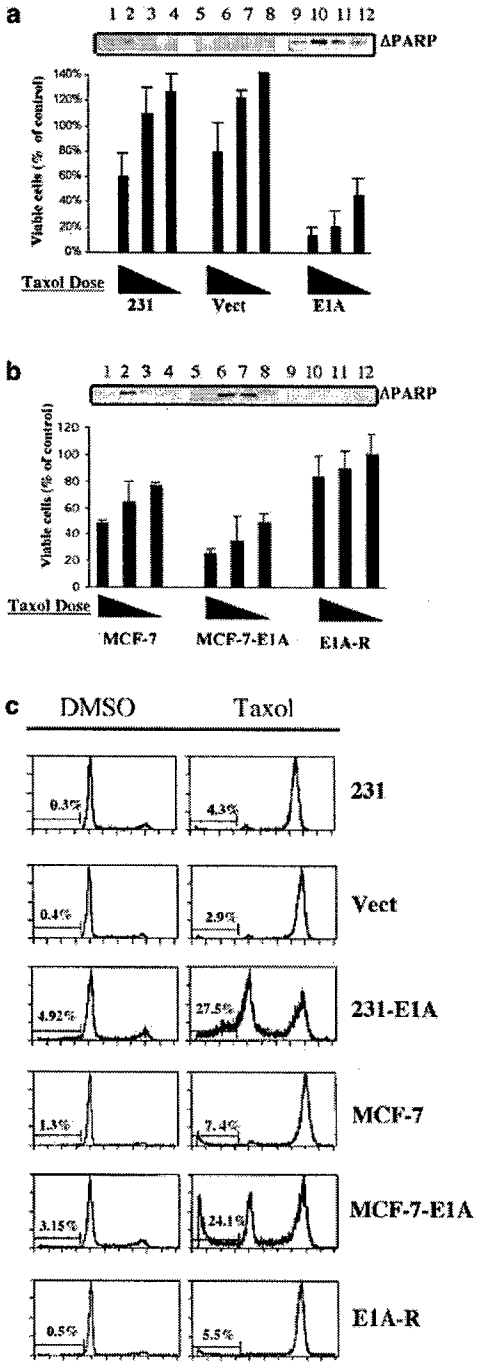
To test whether E1A can sensitize cells to paclitaxel-induced apoptosis in low *Her-2/neu*-expressing cells in cell culture *in vitro*, we treated MDA-MB-231 (p53 mutant) and MCF-7 (p53 wild type) human breast cancer cell lines and their E1A-expressing stable cells with different dosages of paclitaxel and performed MTT cytotoxicity assays. We found that stable expression of wild-type E1A enhanced sensitivity to paclitaxel-induced killing in MDA-MB-231 cells in a dose-dependent manner. Compared with their IC_{50} dosage, the E1A-expressing stable cells were 10 times more sensitive than that of the paclitaxel-treated parental or vector-transfected control cells (Fig 1a). Stable expression of E1A also enhanced the sensitivity of MCF-7 cells to paclitaxel, although to a lesser extent. Interestingly, a revertant of the MCF-7 clone that lost its E1A expression during the long-term *in vitro* cell culture also lost its sensitivity to paclitaxel (Fig 1b). Three additional independent E1A stable clones from each cell line were analyzed, all of which expressed high levels of E1A protein and showed similar sensitivity to paclitaxel (data not shown).

To address whether apoptosis was involved in E1A-mediated sensitization to paclitaxel, we examined the presence of poly (ADP-ribose) polymerase (PARP) cleavage as an apoptotic cell death marker. We detected a dose-dependent increase of PARP cleavage in E1A-expressing MDA-MB-231 (231-E1A) and MCF-7 (MCF-7-E1A) cells upon treatment with 0.1–0.001 μM paclitaxel. In parental and 231-Vect cells, however, PARP cleavage was observed only upon treatment with 0.1 μM paclitaxel, while in the revertant MCF-7 cell clone, it was not detected (Inset, Fig 1a and b). Fluorescence-activated cell sorting (FACS) analysis also supported that E1A enhanced paclitaxel-induced apoptosis, as demonstrated by an increased proportion of sub-G1-phase cells in E1A-expressing cell lines (Fig 1c). Thus, E1A-mediated sensitization to paclitaxel in breast cancer MDA-MB-231 and MCF-7 cells is consistent to the previous report for E1A-mediated sensitization to multiple chemotherapeutic drugs.[26,32]

Expression of E1A enhances paclitaxel-induced antitumor effect in vivo

To test whether E1A also mediated sensitization to paclitaxel *in vivo*, we inoculated stable E1A-expressing (231-E1A) and vector-transfected (231-Vect) MDA-MB-231 cells into mouse m.f.p. In our pilot study, we inoculated the same amount of MDA-MB-231 and 231-E1A cells into mouse m.f.p. and observed that few of the animals with inoculation of the 231-E1A cells developed tumor and even though in some cases, the tumors developed, they are much smaller than those of the MDA-MB-231 cells (data not show). Therefore, in order to ensure tumor formation with comparable size in 231-E1A-inoculated animals, twice the amount of 231-E1A

cells were inoculated. When the tumor established, animals were then treated with or without paclitaxel, via intraperitoneal injection, once a week, for four consecutive weeks. Mice were grown for another 6 weeks and were then killed. Tumor tissues were collected and measured. The mean tumor weight of animals inoculated with 231-E1A cells is significantly lower than that of animals inoculated with 231-Vect cells (Fig 2A, $P<.01$), even though twice the amount of cells were originally inoculated in the 231-E1A group. The result supported



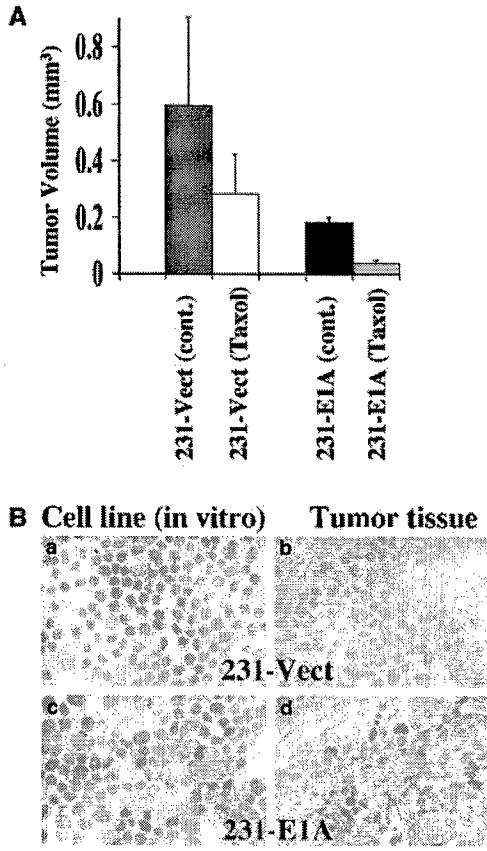


Figure 2 E1A enhanced paclitaxel antitumor effect in nude mice *in vivo*. (**A**) Tumor volume of animals inoculated with MDA-MB-231-Vect (231-Vect) or MDA-MB-231-E1A (231-E1A) stable cells with or without treatment with paclitaxel. At least five animals were included in each group. (**B**) Immunohistochemical analysis of E1A expression in 231-Vect (a,b) and 231-E1A (c,d) cells growing in tissue culture *in vitro* (a, c, respectively) and in tumor tissue samples *in vivo* (b, d, respectively) after cells were inoculated into animal.

the previous reports that expression of E1A alone had a significant effect on repression tumor growth *in vivo* independent of *Her-2/neu* status.[18,33] Treatment with

Figure 1 E1A-mediated sensitization to paclitaxel-induced apoptosis *in vitro*. (**a**) MTT assay. Percentage of viable cells after exposure to 0.1, 0.01, and 0.001 μM of paclitaxel (Taxol) for 24 hours in MDA-MB-231 (231), vector-transfected cells (Vect), and E1A-expressing cells (E1A) detected using MTT assay. The number of viable cells without paclitaxel treatment was defined as 100%. The inset shows a cleaved PARP p89 fragment (ΔPARP) that was detected using a rabbit polyclonal antibody against cleaved PARP. Lanes 1, 5, and 9 in the inset represent cleaved PARP products from cells without paclitaxel treatment. (**b**) Percentage of viable cells after exposure to paclitaxel in MCF-7 cells, MCF-7/E1A-expressing cells (MCF-7-E1A), and a revertant of E1A-expressing clone (E1A-R) detected using MTT assay. The inset shows ΔPARP, and lanes 1, 5, and 9 in the inset represent cleaved PARP products from cells without paclitaxel treamtment. (**c**) FACS analysis of sub-G1 phase apoptotic cells with or without exposure to 0.01 μM paclitaxel for 24 hours. The symbols used are the same as those in panels **a** and **b**. DMSO, dimethyl sulfoxide.

paclitaxel of tumors formed by inoculation of 231-E1A cells achieved 80% reduction of tumor weight in comparison with untreated control 231-E1A tumors. Paclitaxel treatment of tumors formed by 231-Vect cells also achieved nearly 50% reduction of tumor weight. These results indicate that paclitaxel alone is sufficient to achieve a 50% cytoreduction of tumor mass in comparison with mice treated with normal saline (NS) (Fig 2A). Comparing the percentage of tumor shrinkage in paclitaxel-treated 231-Vect tumor group, paclitaxel treatment of 231-E1A tumor achieved a better tumor shrinkage than that of the paclitaxel-treated 231-Vect animals, that is, the 231-E1A group was more sensitive to Taxol ($P<.01$). Thus, expression of E1A also sensitized paclitaxel cytotoxic effect in established tumors *in vivo*.

To test whether E1A was still expressed in tumor tissue *in vivo*, we then analyzed E1A expression in tumor tissue slides and used original 231-E1A stable cells prepared by using cytospun as the positive control by immunohistochemistry. Modest to strong E1A staining was detected in more than 90% of 231-E1A stable cells cultured *in vitro* in the cytospun slides (Fig 2B). However,less than half of the cells were positive for E1A staining in tumor tissue samples obtained from the original 231-E1A inoculated animals, most of them only had modest or weak staining of E1A (Fig 2B). This result suggests that most of the cells with higher E1A expression were lost under the *in vivo* selection pressure during tumor development. None of the control 231-Vect stable cells or 231-Vect inoculated tumors had a positive staining for E1A (Fig 2B). These results also suggest that expression of E1A repressed tumor growth *in vivo*, while loss of E1A expression may result in tumor development in 231-E1A stable cells.

E1A-mediated sensitization to paclitaxel correlated with E1A-induced apoptosis in vivo

To test if E1A-mediated sensitization to paclitaxel cytotoxicity *in vivo* also occurs through apoptotic cell death, we used a TUNEL assay to compare apoptotic index in paclitaxel-treated tumor tissue samples obtained from animals inoculated with 231-E1A cells *versus* those from animals inoculated with 231-Vect cells. In order to detect apoptotic cells *in vivo* following paclitaxel treatment, tumor-bearing animals were treated with paclitaxel (15 mg/kg/animal) once by i.p. injection. Animals were killed 24 hours after paclitaxel treatment and tumor tissue samples were then collected and sectioned for TUNEL assay. In tumor tissues obtained from 231-E1A-inoculated animals ($n=3$), a few TUNEL-positive cells were detected without treatment with paclitaxel; however, treatment with paclitaxel remarkably increased TUNEL-positive cells (from 9 to 18% positive cells). Treatment with paclitaxel also enhanced TUNEL-positive cells in tumor tissue samples obtained from 231-Vect-inoculated animals ($n=3$) (from 3% without paclitaxel to 7% with paclitaxel) (Fig 3A and B). Statistic analysis showed that paclitaxel-treated 231-E1A tumor tissues had a significantly higher percentage of TUNEL-positive cells than did paclitaxel-treated 231-Vect tumor tissues ($P<.001$). Again, the result suggests that expression of E1A significantly enhanced paclitaxel cytotoxicity in tumor tissue *in vivo*. The relatively higher frequency of TUNEL-positive cells detected in tumor tissues obtained from 231-E1A-inoculated animals also suggests that the relatively small tumors formed in 231-E1A-inoculated animals in Figure 2A may in part be due to the enhanced apoptosis in 231-E1A tumors.

Enhancement of paclitaxel-induced antitumor effect by systemic delivery of E1A gene in an orthotopic breast cancer model in vivo

To explore whether E1A could directly enhance paclitaxel-induced killing in a gene therapy setting *in vivo*, we designed systemic E1A gene therapy experiments in mice by i.v. injection of liposome encapsulated E1A gene via the mouse tail vein with or without combination of paclitaxel chemotherapy. MDA-MB-231 cells were inoculated orthotopically into mouse m.f.p. and were allowed to grow until a palpable tumor was formed. When tumors were established, animals were regrouped and randomly assigned to each experimental group. We used SN-liposome formulation as our gene-delivery system because of its relatively high efficiency *in vivo*.[28] SN-liposome (SN) or SN-liposome encapsulated E1A gene was given twice a week for 6 weeks through mouse tail vein, while paclitaxel was given once a week by intraperitoneal injection for 6 weeks. We started to monitor tumor size once a week when animals received each treatment the first time and continued until 6 weeks later after each treatment ended (total for 12 weeks). We then compared the mean tumor volume between each treatment group in order to assess the therapeutic effects of each treatment regime. Compared with control SN-liposome alone, treatment with either systemic delivery of E1A gene or i.p. injection of paclitaxel repressed tumor growth during the 12-week observation period ($P<.05$, $P<.01$) (Fig 4). Compared with treatment of systemic E1A gene alone or paclitaxel alone, a combination of E1A gene therapy and paclitaxel chemotherapy produced significantly enhanced therapeutic effects of paclitaxel and dramatically repressed tumor growth (both $P<.0001$) (Fig 4). In addition, tumors were completely eradicated in four out of seven mice treated with combination therapy. Thus, combination of systemic E1A gene therapy with paclitaxel chemotherapy had a synergistic effect in repressing tumor growth in the orthotopic breast cancer model *in vivo*.

Prolonged animal survival rate by E1A gene therapy in combination with paclitaxel chemotherapy

In order to see if combination of systemic E1A gene therapy with paclitaxel chemotherapy could increase animal survival time, we extended our observation on animals after receiving the above treatments. The end point criteria include that either the tumor size reaches 2 cm in diameter or that animals are moribund. In animals that received treatment with liposome alone, most of the animals died or were killed (because their tumors reached

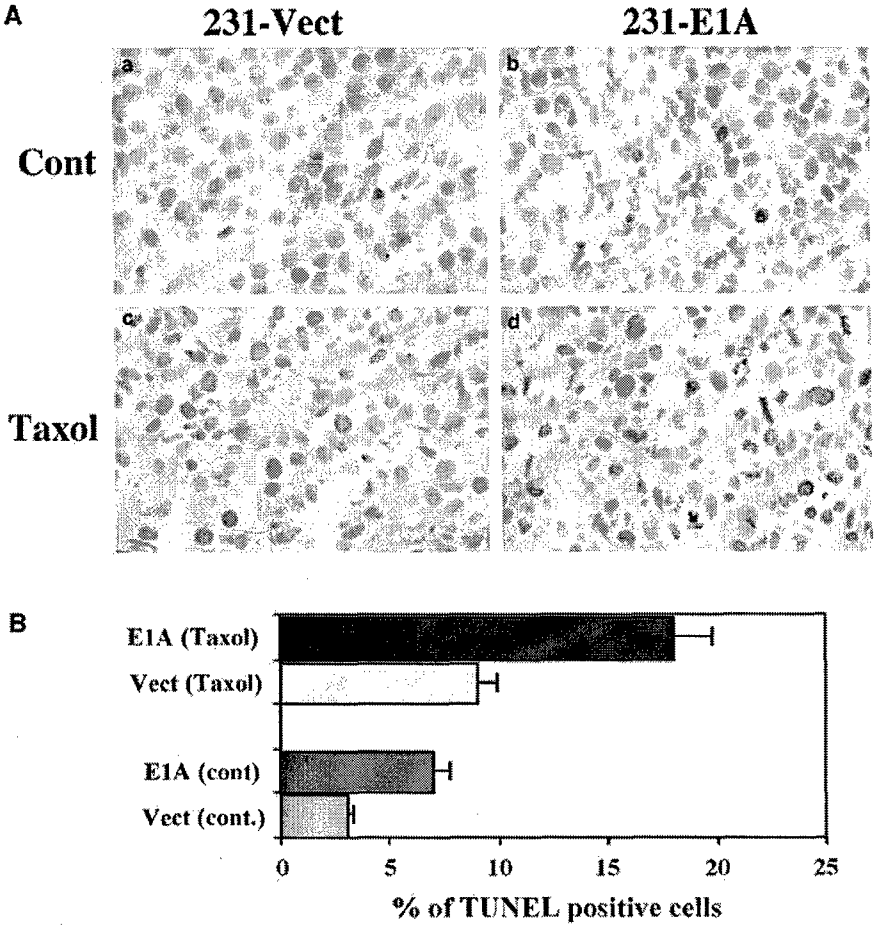


Figure 3 TUNEL assay of apoptotic cells in tumor tissue *in vivo*. (**A**) TUNEL labeling of apoptotic cells in tumor tissue samples obtained from 231-Vect- or 231-E1A-inoculated animals with (b, d) or without (a,c) treatment with paclitaxel. (**B**) The percentage of TUNEL-positive cells in the tumor tissue samples obtained from 231-Vect- and 231-E1A-inoculated animals (each $n=3$) with or without treatment of paclitaxel.

end point criteria) within 3 months after treatment. Although E1A-liposome alone did not dramatically repress tumor growth as that of paclitaxel alone did (Fig 4), treatment with systemic delivery of E1A gene achieved a comparable enhancement than that of the animals treated with paclitaxel alone in terms of prolongation of the animal survival times ($P<.05$) (Fig 5). Compared with survival rate of animals with treatment of SN-liposome, treatment with either E1A gene or paclitaxel had a longer survival time (both $P<.05$). In nearly a year's close observation, we found that combination of systemic E1A gene therapy with paclitaxel chemotherapy achieved a significantly better outcome in animals survival rates, compared with all the three single treatment regimes, including SN-liposome alone, liposome-E1A, or paclitaxel (all $P<.01$) (Fig 5). In addition, three out of seven animals treated with combination therapy achieved over 1 year's tumor-free survival. Thus, the combination of systemic E1A gene therapy with paclitaxel chemotherapy significantly enhanced paclitaxel's antitumor effect and dramatically prolonged animal survival rates in the orthotopic breast cancer model *in vivo*.

Discussion

In the current study, we showed that E1A mediated sensitization to the paclitaxel-induced apoptosis *in vitro* in low *Her-2/neu*-expression breast cancer MCF-7 and MDA-MB-231 cells. We showed that expression of E1A also enhanced the therapeutic effect of paclitaxel in an orthotopic breast cancer model *in vivo*. In addition, we demonstrated that combination of a systemic E1A gene therapy using a surface-protected SN-liposome-E1A complex formulation with paclitaxel chemotherapy significantly enhanced paclitaxel's antitumor effect and dramatically prolonged animal survival rates in an orthotopic breast cancer animal model. Our data showed that the combination of both E1A gene therapy and paclitaxel chemotherapy is much more efficient than either E1A gene therapy or paclitaxel alone in terms of repression of tumor growth *in vivo* and prolongation of animal survival rate. Unlike the trials on *Her-2/neu* overexpression tumors that used *Her-2/neu* expression as the marker, test on low *Her-2/neu* expression tumors may need a surrogate marker or markers in order to monitor whether the therapy works in this subgroup of tumors. In

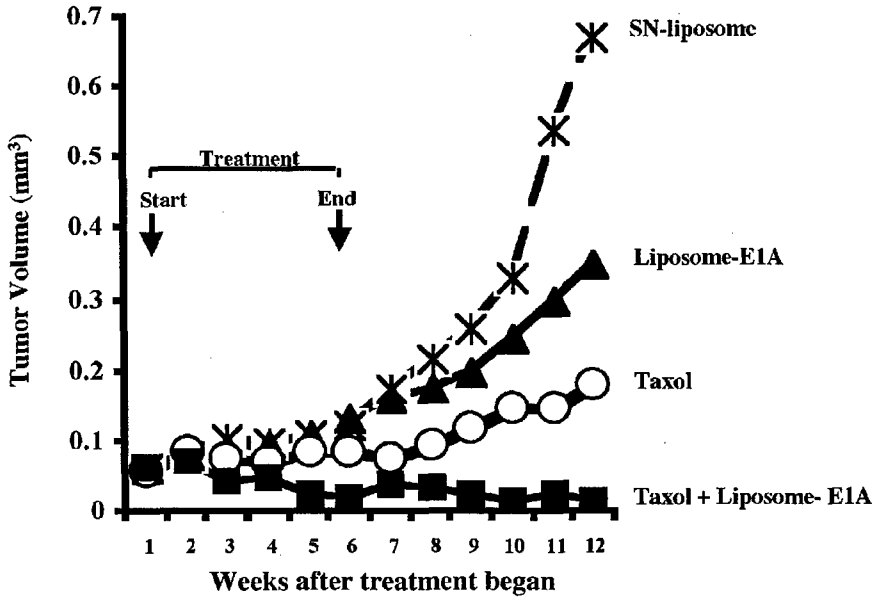


Figure 4 Tumor volume of animals during or after treatment with systemic delivery of liposome-E1A alone with or without i.p. injection of paclitaxel. Treatment groups included SN-liposome vehicle (✳), SN-liposome-E1A (▲), paclitaxel alone (○), or paclitaxel plus SN-E1A (■). At least seven animals were included in each group.

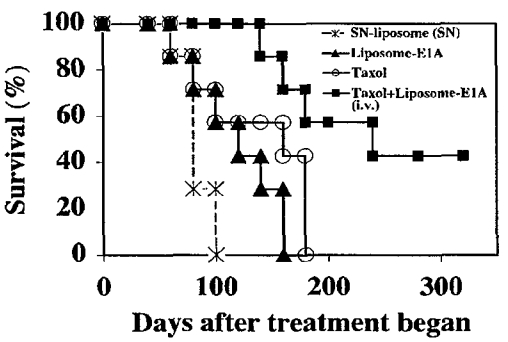


Figure 5 Survival curve of animals after treatment with systemic delivery of SN-liposome alone (✳), i.v. liposome-E1A alone (▲), i.p. injection of paclitaxel (○), or combination of systemic liposome-E1A and i.p. injection of paclitaxel (■).

the literature, previous studies on E1A-mediated sensitization to drug-induced apoptosis in different cell systems or cell-free systems have identified a few other molecules that are also linked to E1A-mediated chemosensitization other than downregulation of *Her-2/neu*, such as the proapoptotic protein Bax, Apaf-1, p19ARF, procaspase-2, -3, -7, -8, and -9, cell cycle inhibitor $p21^{Cip1/Waf-1}$, or as yet unidentified inhibitor that ordinarily provides protection against cell death.[23,32,34–41] Whether one of these molecules or other unknown molecules could be used as a surrogate marker to monitor the therapeutic effect of this combination therapy in tumors with low *Her-2/neu* expression needs further assessment.

It is worthwhile mentioning the important clinical implications of the concentration of paclitaxel that we tested in the *in vitro* study because the paclitaxel concentration that can kill cancer cells *in vitro*, in the presence of E1A, is clinically relevant. A plasma concentration of 5–10 μM paclitaxel can be achieved after bolus infusion of paclitaxel, but it rapidly falls to a level of several hundred nanomolar or less.[42] Our experiments showed that 10 nM paclitaxel was sufficient to induce apoptosis *in vitro* in E1A-expressing cells but not in parental or vector control cells (Fig 1). This indicates that a clinically relevant concentration (5 ~ 200 nM) of paclitaxel is sufficient to kill E1A-expressing cells, but parental cells require much higher dosage, which may be difficult to be achieved in a clinical setting.[42]

We have previously shown that *Her-2/neu*-overexpressing cells are resistant to paclitaxel-induced apoptosis[43,44] and E1A, through downregulation of *Her-2/neu*, can sensitize cellular response to paclitaxel-induced apoptosis.[22,23,43,44] In those studies, we could not detect E1A-mediated chemosensitization in the low *Her-2/neu*-expressing cells.[22] The major reason for this discrepancy was due to the paclitaxel concentrations tested. The *Her-2/neu*-overexpressing cancer cells are resistant to paclitaxel even at a dosage of 10.0 μM, in the presence of E1A, they became sensitive even at the dose of 1.0 μM of paclitaxel.[22,23] The low *Her-2/neu*-expressing cells, such as MDA-MB-231and MDA-MB-435, are much more sensitive to paclitaxel, even at paclitaxel concentration of 0.1 μM they are still sensitive.[22] Therefore, in the previous studies, the dose of paclitaxel was too high (1 μM) to detect E1A-mediated paclitaxel sensitization that became detectable in the present study (0.01 μM). Thus, the E1A gene therapy strategy may represent a novel and unique way to

enhance the sensitivity of tumor cells to chemotherapy and the current study provides solid preclinical data that may help in developing further clinical trials using the combination of chemotherapy with systemic gene therapy.

Acknowledgments

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Advances in Brief

A New Role of Protein Phosphatase 2A in Adenoviral E1A Protein-Mediated Sensitization to Anticancer Drug-Induced Apoptosis in Human Breast Cancer Cells

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Abstract

The adenoviral type 5 E1A protein has been shown to induce sensitization to different categories of anticancer drug-induced apoptosis, partly by down-regulation of the activity of a critical oncogenic kinase Akt in both normal fibroblasts and epithelial breast cancer cells. Currently, the adenoviral E1A gene is being tested as an antitumor gene in multiple clinical trials. However, molecular mechanisms underlying E1A-mediated chemosensitization and down-regulation of Akt activity are still not completely defined. Here, we show that E1A by up-regulation of the catalytic subunit of protein phosphatase 2A [PP2A (PP2A/C)] enhanced the activity of PP2A, which results in repression of Akt activation in E1A-expressing cells. In addition, activation of PP2A/C is required for E1A-mediated sensitization to drug-induced apoptosis, because blocking PP2A/C expression using a specific small interfering RNA against PP2A/C reduced drug sensitivity in E1A-expressing cells. Deletion mutation of the conserved domain of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, also abolished the ability of E1A to up-regulate PP2A/C. Thus, the up-regulation of PP2A may represent a novel mechanism for E1A-mediated sensitization to anticancer drug-induced apoptosis.

Introduction

Reversible phosphorylation of proteins by protein kinases and phosphatases is a key regulatory mechanism in the control of multiple cellular processes, ranging from cell proliferation and survival to cell death. Many oncogenes identified today are protein kinases; because their kinase activities are finely regulated by respective protein phosphatases, it is important to understand how the respective protein phosphatases are involved in the regulation of these cellular processes (1). Thus far, alterations or mutations of very few phosphatases have been implicated in the development of tumors, and only the dual-specific protein phosphatase PTEN exhibits many characteristics of a typical tumor suppressor (2, 3). A tumor-suppressive function of protein phosphatase 2A (PP2A) has been proposed, because a deletion mutation of the regulatory subunit of PP2A was found in primary human breast, colon, and lung tumors and melanoma (2) and inactivation of PP2A by the small t antigen of the DNA tumor virus SV40 was also found to be involved in viral-induced cell transformation (4). The core enzyme of PP2A is a dimer, consisting of a catalytic subunit (PP2A/C) and a regulatory or structural A subunit (PP2A/A). A third regulatory B subunit (PP2A/B), which determines substrate specificity, can be associated with this core structure (1, 2). Recent evidence indicates that PP2A forms stable complexes with protein kinase signaling molecules, indicating that it plays a central, regulatory role in signal transduction mediated by reversible protein phosphorylation (1). Although the role of PP2A in the regulation of apoptosis is not clear, results from a gene knockout study of PP2A/C imply that it may play a critical role in regulation of apoptotic signaling (5). In supporting of this notion, several groups reported that PP2A through the dephosphorylation of a key oncogenic survival factor Akt participated in the regulation of apoptosis induced by ceramide, mistletoe lectin, and 4-hydroxynonenal, an aldehyde product of membrane lipid peroxidation (6–8).

Recently, we and other groups have shown that repression of Akt activation by adenoviral E1A contributed to E1A-mediated sensitization to anticancer drug-induced apoptosis in both normal fibroblast and epithelial breast cancer cells (9–11). However, the mechanism involved in E1A-mediated repression of Akt activity is still not known. Here, we show that the PP2A phosphatase activity is enhanced in E1A-expressing cells through E1A-mediated up-regulation of PP2A/C expression, which results in repression of Akt activation. We demonstrated that PP2A is involved in regulation of apoptosis and that activation of PP2A/C is also required for E1A-mediated sensitization to anticancer drug-induced apoptosis in E1A-expressing breast cancer cells.

Materials and Methods

Cell Lines. The stable E1A-expressing cell lines and domain deletion mutants were established as described previously (10).

Preparation of Cell Lysates, Western Blot Analysis, and Antibodies. Preparation of cell lysates and Western blot analysis were performed according to standard protocols as described previously (10). Rabbit antihuman PP2A/A and PP2A/C were purchased from CalBiochem (La Jolla, CA). Information of other antibodies used was described previously (10).

Serine/Threonine Phosphatase Assay. A nonradioactive serine/threonine phosphatase assay system was purchased from Promega Corporation (Madison, WI). PP2A phosphatase activity was measured according to the manufacturer's protocol.

Immunoprecipitation, Protein Phosphatase 2A Treatment, and Akt Dephosphorylation Assay. To measure endogenous Akt dephosphorylation by exogenous purified recombinant human PP2A (Upstate Biotechnology, Lake Placid, NY), cells were stimulated using 10 μmol/L insulin for 15 minutes before harvesting. To measure exogenous Akt dephosphorylation, Hemagglutinin-tagged Akt was transiently transfected into 293T cells. Cells were lysed and immunoprecipitated with anti-Akt or anti-HA antibodies. Aliquots of the Akt immunoprecipitates were incubated with various doses of recombinant human PP2A at 30°C for 30 minutes, after which the reaction was terminated through the addition of 6× SDS loading buffer and resolved using 10% SDS-PAGE. Phosphorylated and total Akt and HA-tag were measured using anti-phospho-Akt (Thr-308-p) and total Akt and anti-HA antibody, respectively.

Synthesis and Application of Small Interfering RNA. Double-stranded small interfering RNAs were produced *in vitro* using chemically synthesized

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DNA oligonucleotide templates (Sigma, St. Louis, MO) and the T7-MEGA-shortscipt kit (Ambion, Inc., Austin, TX) according to the T7 small interfering RNA protocol described by Paddison *et al.* (12). The specific primer sequences for PP2A/C are as follows: A, 5′-CCG AGT CCC AGG TCA AGA G CC TAT AGT GAG TCG TAT TAC-3′; and B, 5′-GAG GCT CTT GAC CTG GGA C CC TAT AGT GAG TCG TAT TAC-3′. The nonspecific scrambled control primer sequences are as follows: A, 5′-ATG GAG AGC AGG TCA AAC T CC TAT AGT GAG TCG TAT TAC-3′; B. 5′-TTG GAG TTT GAC CTG CTC T CC TAT AGT GAG TCG TAT TAC-3′.

Statistical Analysis. Statistical analysis was performed with a two-tailed Student's *t* test, and $P < 0.05$ was considered statistically significant.

Results and Discussion

Protein Phosphatase 2A Activity Is Enhanced, Which Is Correlated with Elevated Expression of the Catalytic Subunit of Protein Phosphatase 2A in Stable E1A-Expressing Cells. Phosphorylation of protein kinases are tightly regulated by related protein phosphatases, and two phosphatases, PTEN and PP2A, have been shown to repress Akt activation through dephosphorylation (3, 6–8). To identify whether protein phosphatases were involved in E1A-mediated down-regulation of Akt activation, we measured the alteration of protein phosphatases, such as PTEN and PP2A, in stable E1A-expressing cells *versus* that in vector control cells. We did not detect any change in PTEN expression in stable E1A-expressing cells *versus* control cells (data not show). Also, there was no change in the expression level of the PP2A regulatory A subunit PP2A/A, however, we did detect elevated expression of the catalytic subunit of PP2A (PP2A/C) in multiple stable E1A-expressing cells (Fig. 1*A*). Thus, we further tested whether PP2A activity was increased in the E1A-expressing cells by using a specific PP2A phosphatase assay. We observed that the PP2A activity was enhanced in E1A-expressing MDA-MB-231 cells (231-E1A) in a dose (protein and substrate concentration)-dependent manner compared with that of the vector control cells (231-Vect; both $P < 0.01$; Fig. 1*B*). The above results suggest that E1A, by up-regulating PP2A/C expression, enhances the activity of PP2A.

PP2A Is Involved in the Regulation of Akt and p38 Activities. Next, we asked whether enhanced PP2A activity in E1A-expressing stable cells contributed to E1A-mediated repression of Akt activation. First, we verified whether Akt could be dephosphorylated by recombinant human PP2A, which contains both the catalytic and the regulatory A subunits. Dephosphorylation of endogenous Akt occurred in a recombinant human PP2A dose-dependent manner (Fig. 1*C*). Similar to the dephosphorylation of endogenous Akt, the HA-tagged, exogenous Akt was also dephosphorylated by recombinant human PP2A in a dose-dependent manner *in vitro* (data not show). To test whether dephosphorylation of Akt is dependent on PP2A activity, we used the specific PP2A inhibitor okadaic acid to block PP2A activity and measured Akt phosphorylation in the presence of recombinant human PP2A. We found that dephosphorylation of both endogenous and exogenous Akt was completely abolished in the presence of 1 nM PP2A inhibitor okadaic acid (Fig. 1*C*; data not show). This result suggests that

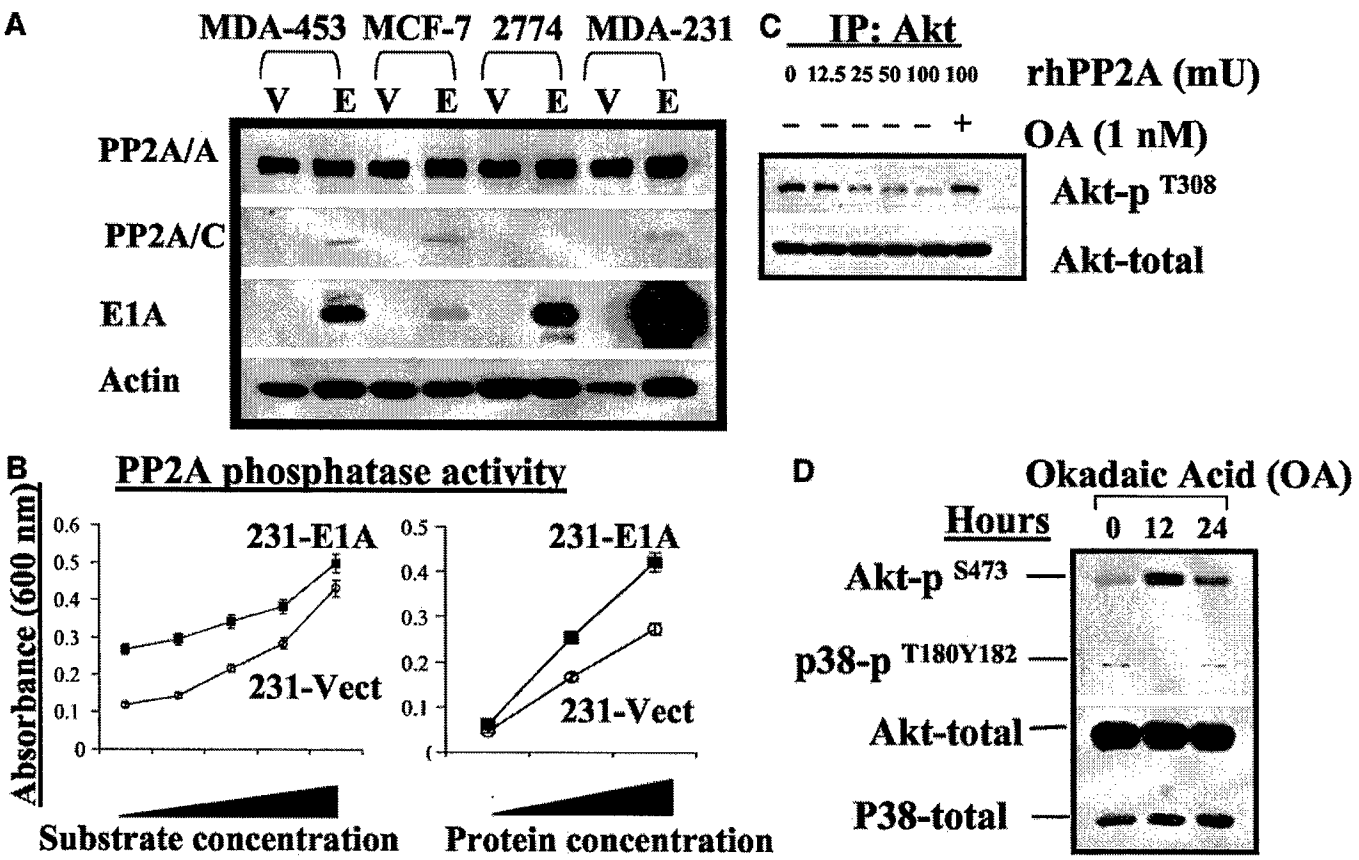

Fig. 1. PP2A activity is enhanced in stable E1A-expressing cells through up-regulation of PP2A/C. *A*, Protein expression of PP2A/A, PP2A/C, E1A, and actin in stable E1A-expressing cells (*E*) and corresponding vector controls (*V*). *B*, PP2A activity was measured using a protein phosphatase assay kit. The substrate phospho-peptide concentrations were 20, 40, 100, 200, and 400 μmol/L, respectively. Protein concentrations include 0.1, 1.0, and 10 μg, respectively. *V*, 231-Vector. *E*, 231-E1A. Results shown here are from free independent experiments. *C*, Dephosphorylation of Akt by purified hPP2A (1 milliunit = 1×10^{-3} units) and inhibition of by okadaic acid (1 nmol/L) *in vitro*. Aliquots of immunoprecipitated endogenous Akt were incubated with purified human PP2A enzyme (*rhPP2A*); relative phosphatase activities were measured by anti-phospho-Akt (*T308*) antibody. Total Akt was used as a loading control. *D*, Blocking PP2A activity by exposing 231-E1A cells to okadaic acid (10 nmol/L) increased Akt phosphorylation and inhibited p38 phosphorylation.

dephosphorylation of Akt is mediated by PP2A and dependent on PP2A phosphatase activity *in vitro*.

To test whether dephosphorylation of Akt also occurs in cells *in vivo*, stable E1A-expressing 231-E1A cells were treated with okadaic acid, and the Akt phosphorylation level was monitored for 24 hours. In the presence of okadaic acid, the Akt phosphorylation level was significantly increased at 12 hours, and the increment was subsequently reduced at 24 hours, presumably due to the limited half-life of okadaic acid (Fig. 1*D*). Previously, we and other groups have shown that activation of Akt results in inactivation of p38 (10, 13), therefore we also measured p38 phosphorylation before and after exposure with okadaic acid. Inconsistent with the previous studies, phosphorylation of p38 was detected before exposure to okadaic acid (0 hours) and was repressed at 12 hours when Akt phosphorylation was increased. Additionally, p38 phosphorylation was recovered at 24 hours when accumulation of Akt phosphorylation was reduced (Fig. 1*D*). Both total Akt and total p38 protein levels had no change throughout exposure with okadaic acid (Fig. 1*D*). This result indicates that both Akt and p38 phosphorylation can also be regulated by PP2A *in vivo*.

Up-regulation of Protein Phosphatase 2A/C Is Required for E1A-Mediated Chemosensitization. It has been reported that E1A could sensitize anticancer drug-induced apoptosis through down-regulation of Akt activation (9, 10); we asked whether E1A-induced up-regulation of PP2A/C might play a role in E1A-mediated chemosensitization. To test this, we measured PP2A/C expression during anticancer drug-induced apoptosis in both 231-Vect cells and 231-E1A cells. We used poly(ADP-ribose) polymerase (PARP) cleavage as a marker of apoptosis and Bcl-2 phosphorylation as an indication of the pharmaceutical effect of the antimicrotubule drug paclitaxel (14). The protein level of PP2A/C was further increased and was correlated with reduced Akt phosphorylation, increased p38 phosphorylation and Bcl-2 phosphorylation, and increased PARP cleavage after exposure

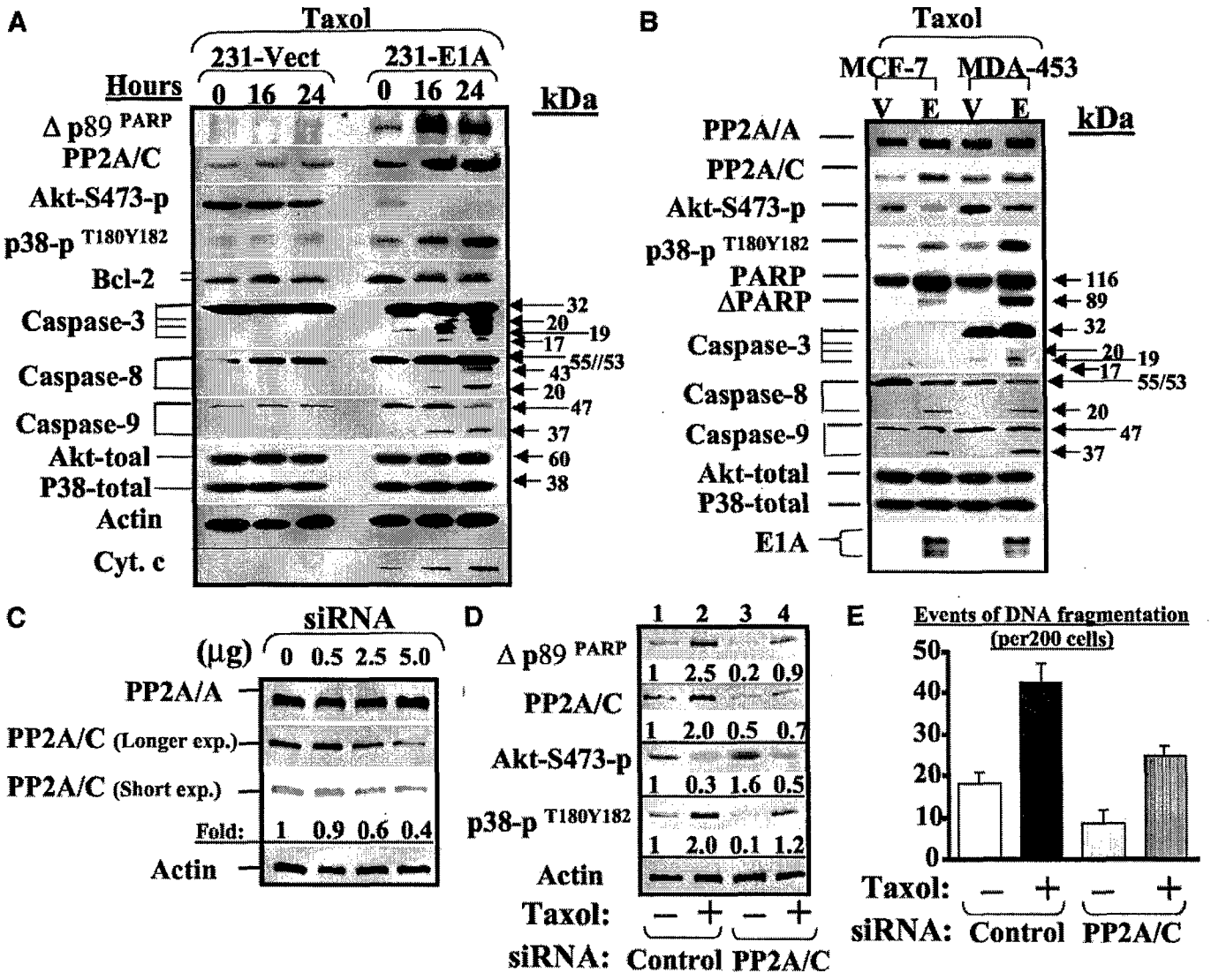

Fig. 2. Up-regulation of PP2A/C is required for E1A-mediated chemosensitization. *A*, PARP cleavage and PP2A/C activation in 231-Vect and 231-E1A cells after treatment with paclitaxel. *Cyt. c*, cytochrome *c*. *B*, Western blot analysis of PP2A and the catalytic subunit PP2A/C in Vect (*V*)- or E1A (*E*)-transfected MCF-7 and MDA-MB-453 cells after exposure to 0.01 and 1.0 μmol/L paclitaxel, respectively. *C*, a dose-finding test of specific PP2A/C small interfering RNA on PP2A/C expression in 231-E1A cells. Relative intensity of PP2A/C was shown in the *bottom*. *D*. Cells were transfected with small interfering RNA using JetSI cationic transfection reagent (Obiogene, Inc., Carlsbad, CA) for 16 hours and replaced with fresh medium before addition of 0.01 μmol/L paclitaxel and were incubated for another 24 hours before harvesting. Relative intensities of PARP and PP2A/C in specific small interfering RNA (*PP2A/C*) and nonspecific control small interfering RNA (*Control*) protected cells were shown in the *bottom* of each *band*. *E*, For nuclear fragmentation analysis, cells were grown in Lab-Tek Chamber Slides (Nunc, Inc., Naperville, IL) and were treated by the same procedure as described above. Cells were then washed with PBS twice, fixed with 70% alcohol, and stained with Hoechst 33342 (0.5 μg/mL; Sigma). Events of apoptotic nuclei were counted under a fluorescence microscope, and the mean values in every 200 cells in each field were plotted.

to paclitaxel in the 231-E1A cells, suggesting that increased PP2A/C is correlated with drug-induced apoptosis in E1A-expressing cells (Fig. 2*A*). In addition, to test which apoptotic pathway is involved in E1A-mediated sensitization to paclitaxel-induced apoptosis, we measured the expression of caspase-3, -8, -9, and cytosolic fraction of cytochrome *c* (15). We observed that paclitaxel-induced PP2A/C expression and PARP cleavage in 231-E1A cells correlated with cytochrome *c* release and activation of procaspase-8, -9, and -3. This result suggests that both intrinsic (represented by cytochrome *c* release and caspase-9 cleavage) and extrinsic (represented by activation of procaspase-8) apoptotic pathways may be involved in E1A-mediated sensitization to paclitaxel-induced apoptosis (15). PARP cleavage and increased PP2A/C was also observed in the 231-Vect cells after treatment with paclitaxel, but to a much lesser extent, it implied that PP2A/C may be required for drug-induced apoptosis in the absence of E1A.

To test whether the above observation can also be applied to E1A-mediated chemosensitization in other cell lines, additional two pairs of E1A-expressing stable cell lines and the vector DNA-transfected controls were tested. We observed notably higher levels of PP2A/C, reduced Akt phosphorylation, enhanced p38 phosphorylation, and cleaved PARP fragment in E1A-expressing cells after exposure to paclitaxel compared with that of the corresponding vector controls. Again, enhanced expression of PP2A/C and cleaved PARP fragment in E1A-expressing cells after exposure to paclitaxel correlated with the activation of procaspase-3, -8, and -9 (Fig. 2*B*), whereas the expression level of the regulatory subunit of PP2A/A was not significantly altered in E1A-expressing cells *versus* control cells (Fig. 2*B*). These data suggest that elevated expression of PP2A/C in E1A-expressing cells is involved in E1A-mediated sensitization to drug-induced apoptosis.

To further test whether up-regulation of PP2A/C expression by E1A is required for E1A-mediated sensitization to apoptosis, we used a double-stranded small interfering RNA against PP2A/C as a tool to knockdown PP2A/C expression in E1A-expressing cells. First, we did a dose escalation study of small interfering RNA on PP2A/C protein expression. We found 5 μg of small interfering RNA were sufficient to repress PP2A/C expression (~60% reduction in PP2A/C protein expression) in 231-E1A cells (Fig. 2*C*). Second, we tested whether repression of PP2A/C expression would also inhibit drug-induced apoptosis in E1A-expressing cells. We exposed 231-E1A cells to either a specific small interfering RNA against PP2A/C or a scrambled, nonspecific control small interfering RNA in the presence or absence of paclitaxel for 24 hours. Third, we measured the expression levels of cleaved PARP and PP2A/C and counted events of nuclear fragmentation under microscopy as an alternative measure for the occurrence of apoptotic cells. We detected a 2-fold increase in the expression level of PP2A/C and 2.5-fold increase of cleaved PARP in the presence of paclitaxel compared with cells treated with control small interfering RNA alone without paclitaxel (Fig. 2*D*, *Lanes 1 versus 2*). However, when compared with cells in the presence of control small interfering RNA, cells treated with PP2A/C-specific small interfering RNA had reduced both PP2A/C expression and cleaved PARP (0.5-fold of PP2A/C and 0.2-fold cleaved PARP proteins; Fig. 2*D*, *Lanes 1 versus 3*). In the presence of both PP2A/C-specific small interfering RNA and paclitaxel, the increment of PP2A/C expression is minimal (0.7-fold that of control small interfering RNA alone) and the cleaved PARP protein is comparable with control small interfering RNA alone (0.9-fold) in the absence of paclitaxel (Fig. 2*D*, *Lanes 1 versus 4*). Inconsistent with the above results, we also observed that when PP2A/C expression was blocked by specific small interfering RNA, Akt phosphorylation was elevated, whereas p38 phosphorylation was reduced (Fig. 2*D*). Corresponding with the expression of PP2A/C and cleaved PARP proteins, the rate of DNA fragmentation in control small interfering RNA-treated cells is about 9% (18 of 200) in the absence of paclitaxel and 21% (42 of 200) in the presence of paclitaxel, whereas in PP2A/C-specific small interfering RNA-treated cells, the rate of DNA fragmentation is about 4% (8 of 200) in the absence of paclitaxel and 12% (24 of 200) in the presence of drug (Fig. 2*E*).

Taken together, the above results suggest that up-regulation of PP2A/C is required for E1A-mediated sensitization to drug-induced apoptosis.

A Protein Phosphatase 2A Phosphatase Activity Is Also Involved in Apoptosis Induced by Different Apoptotic Stimuli, Such as Tumor Necrosis Factor-α. The above results established that by regulation of the Akt activation, PP2A played a role in adenoviral E1A-mediated sensitization to anticancer drug-induced apoptosis by repressing Akt activation and up-regulation of p38 activation. In the previous report, we have shown that repression of Akt activation and up-regulation of p38 activation contributed to different apoptotic stimuli-induced apoptosis, such as exposure to tumor necrosis factor (TNF)-α (10). To further test whether PP2A plays a general role in the regulation of apoptosis by different apoptotic stimuli in the absence of E1A, we treated MDA-MB-231 cells with TNF-α at a dose that could induce apoptosis (10). PP2A phosphatase activity was measured after treatment. As experimental controls, we also treated MDA-MB-231 cells with insulin-like growth factor-1, which is known to transiently stimulate the phosphatidylinositol 3′-kinase-Akt pathway, and MEK inhibitor PD58098, which is not supposed to affect Akt or p38 phosphorylation. We again used the cleaved PARP fragment (p89PARP) as a marker for apoptosis. As expected, we detected cleaved PARP in MDA-MB-231 cells after treatment with TNF-α for 24 hours, correspondingly, we detected increased p38 phosphorylation and reduced Akt phosphorylation. Interestingly, we also observed increased PP2A phosphatase activity after treatment with TNF-α (Fig. 3). However, treatment with the MEK inhibitor PD58098 did not induce detectable PARP cleavage, alteration of Akt and p38 phos-

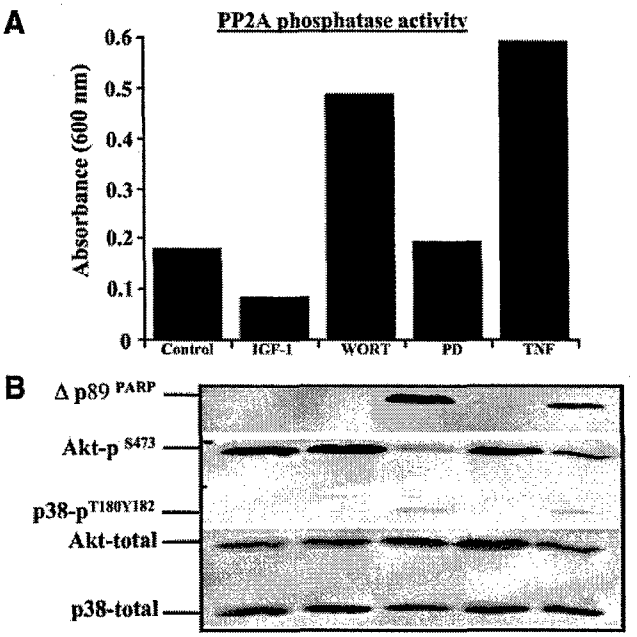

Fig. 3. Enhanced PP2A phosphatase activity in TNF-α induced apoptosis. PP2A phosphatase activity and Western blot analysis of the expression of phospho-Akt, phospho-p38, and PARP cleavage in MDA-MB-231 cells with treatment of insulin-like growth factor-1 (50 ng/mL), TNF-α (50 ng/mL), the phosphatidylinositol 3′-kinase inhibitor Wortmannin (*WORT*; 0.5 μmol/L), and the MEK1/2 inhibitor PD98058 (*PD*; 20 μmol/L).

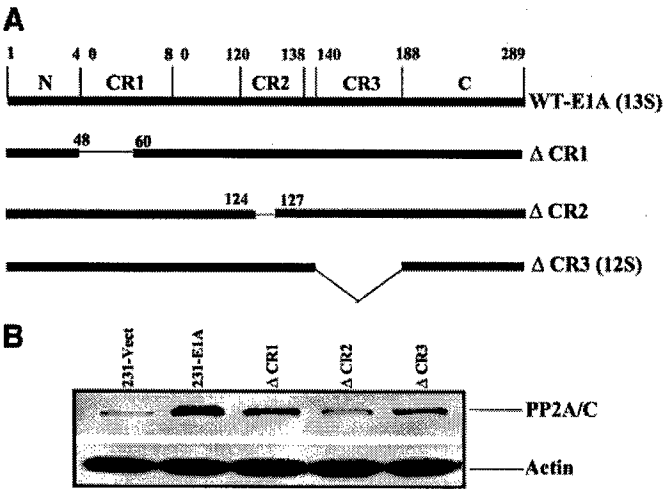

Fig. 4. Conserved domains of E1A required for up-regulation of PP2A/C. *A*, a domain structure and map for deletion mutation of CR1 and CR2. *B*, Western blot analysis of PP2A/C in vector-transfected, or wild-type (*WT*) E1A and mutant E1A (Δ*CR1*, Δ*CR2*, or Δ*CR3*). Actin was used as a loading control.

phorylation, or alteration of PP2A activity. Although insulin-like growth factor-1 did not dramatically affect Akt or p38 phosphorylation at the 24-hour time point, it slightly reduced PP2A activity (Fig. 3). These results suggest that PP2A may also be involved in TNF-α-induced PARP cleavage and apoptosis. In addition, when Akt activity was blocked by phosphatidylinositol 3′-kinase inhibitor wortmannin, PP2A activity was increased, which correlated with decreased Akt phosphorylation, increased p38 phosphorylation, and PARP cleavage (Fig. 3). Additionally, blocking Akt activation using another phosphatidylinositol 3′-kinase inhibitor, LY249002, also increased PP2A activity (data not shown). Thus, the PP2A activity is also involved in apoptosis induced by the blockade of phosphatidylinositol 3′-kinase-Akt pathway.

A Conserved Domain of E1A Is Required for Up-Regulation of Protein Phosphatase 2A/C and Sensitization to Drug-Induced Apoptosis. Because PP2A/C is required for sensitization to drug-induced apoptosis, we then asked whether a deletion mutation of any conserved domain (CR) of E1A, which is required for E1A to sensitize cells to drug-induced apoptosis, also disrupts the ability of E1A to up-regulate PP2A/C expression. In our previous report, we had mapped that among the three conserved domains of E1A, CR2 is associated with E1A-mediated sensitization to drug-induced apoptosis (10). Therefore, we used the same set of wild-type E1A or conserved domain deletion mutant stable cells (ΔCR1, ΔCR2, and ΔCR3) established in MDA-MB-231 cells to test whether the same deletion mutation would affect E1A-mediated up-regulation of PP2A/C (Fig. 4*A*). As expected, we found that deletion mutation of the CR2 domain impaired the ability of E1A to up-regulate PP2A/C expression, whereas the CR1 or CR3 domain mutant only slightly affected E1A-mediated up-regulation of PP2A/C in the presence of paclitaxel (Fig. 4*B*). Thus, this result further supports the requirement of E1A-mediated chemosensitization for up-regulation of PP2A/C.

Taken together, in the current study, we showed that E1A-mediated sensitization to drug-induced apoptosis involves activation of PP2A through up-regulation of PP2A/C expression, which results in activation of p38 and repression of Akt. In addition, activation of PP2A/C is required for E1A-mediated sensitization to drug-induced apoptosis, because blocking PP2A/C expression by a small interfering RNA against PP2A/C reduced drug sensitivity of E1A-expressing cells. Deletion mutation of the conserved domain of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, abolished the ability of E1A to up-regulate PP2A/C (Fig. 4*B*) and down-regulate Akt activation (10). Thus, by repressing Akt activation through PP2A, E1A up-regulates p38 and facilitates cytochrome *c* release from mitochondria (Fig. 2*A*), which, in turn, contributes to E1A-mediated sensitization to drug-induced apoptosis.

Acknowledgments

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April 15, 2005

Yong Liao, Ph.D.
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Houston, TX 77030
713-794-0209

Re: Official Notice of Award, Grant Number BCTR0504146

Dear Dr. Liao:

Congratulations! The Susan G. Komen Breast Cancer Foundation is pleased to announce the grant application you submitted to the Foundation entitled ***Wip1 As A Novel Therapeutic Target For Human Breast Cancer*** has been approved for funding in the amount of $250,000. To expedite the award process, we ask that your Administrative Officer thoroughly review the 2004-2005 Policies and Procedures and Letter of Agreement which are currently on the Komen website at www.komen.org under Grants and Funding/Research Grants/Information for Grantees/2004-2005 Policies and Procedures. The language in these documents is non-negotiable. The document on the website is merely a sample. The official grant agreement will be sent by the Foundation after the award has been accepted by the grantee.

We have enclosed a Research Acceptance Form and Grantee Information Sheet to assist us in accurately preparing your grant agreement. Please print legibly and have an official in your sponsored programs office sign the completed forms and return via fax **to my attention at (972) 855-1640 by April 22, 2005**. When we receive the attached forms, we will forward you the Foundation's "Policies and Procedures for Research and Training Grants 2004-2005 Grant Cycle", which contains a grant agreement for signature. Provided we receive a fully executed grant agreement, your grant award will be effective as of May 1, 2005.

For information purposes only, the rating and reviewer comments will be available to you through our website in May 2005, at www.komen.org/Grants and Funding. Please follow the instructions posted to the web site at that time on how to obtain your comments.

Again, congratulations. We are very excited about this grant and the work that will be accomplished in fulfilling the mission of the Komen Foundation – *to eradicate breast cancer as a life threatening disease by advancing research, education, screening and treatment.*

Sincerely,

Dianne R. McDonald
Research Grants Manager

Liao, Y | BCTR0504146

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Master Application Packet Cover Page

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ELECTRONIC ABSTRACTS DUE AUGUST 2, 2004
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LAY AND SCIENTIFIC ABSTRACTS

DO NOT EXCEED THE DESIGNATED SPACE.

State the background, objective/hypothesis, and specific aims of the proposed project. Describe the design and methodology of the study and conclude with the potential outcomes and benefits of the research.

Abstract Title:
Wip1 As A Novel Therapeutic Target For Human Breast Cancer.

LAY ABSTRACT:

Wip1 As A Novel Therapeutic Target For Human Breast Cancer. Breast cancer is the most common cancer in women worldwide and accounts for one sixth of cancer death in the United States. Significant progress has been made in understanding the biology of breast cancer and in developing new therapies based on this knowledge in the last two decades. However, there is still much to be learned about how breast cancers develop and how to improve the therapeutic efficacy. In addition, there is still a great need for innovative and rationally designed, mechanism-based approach for the treatment of breast cancer. The Wip1 gene is located in the chromosome 17q23 and amplification of 17q23 has been linked to a poor prognosis in breast cancer patients. The overexpression of Wip1 protein has been found in 16% of primary breast tumors. In addition, studies have shown that overexpression of Wip1 repressed a key tumor suppressor p53 functional activation and expedite tumor formation in vivo, while mouse embryo fibroblast deprived Wip1 expression had a reduced growth rate and enhanced p53 functional activity. These findings strongly suggest that high levels of Wip1 contribute to the development of breast cancer and Wip1 may provide a clinically important target for the development of anticancer therapeutics. Thus, reducing the Wip1 level may be a way to reduce the aggressiveness of breast tumors that contain high level of this protein. In addition, reducing the Wip1 level might also make such tumors more sensitive to therapeutics. Our proposed study is designed to find out whether reducing the Wip1 level in breast tumors with high level of this protein makes these tumors less aggressive but more sensitive to therapeutics. Our findings may lead to better understanding of how breast cancer develops and could result in new treatments for breast cancer.

SCIENTIFIC ABSTRACT:

Wip1 As A Novel Therapeutic Target For Human Breast Cancer (BCTR0504146). BACKGROUND: Overexpression of a wild type p53-inducible serine/threonine protein phosphatase, Wip1, (also know as PPM1D), through gene amplification has recently been found in 16% of human breast tumor tissue samples. The Wip1 gene is located in the 17q23 amplicon, a novel region of amplification in breast tumor that has been linked to a poor prognosis in breast cancer patients. Overexpressed Wip1 can expedite tumor formation in cooperation with an activated H-ras or Her-2/neu oncogene both in vitro and in vivo. However, it is not yet know whether overexpression of Wip1 by itself contributes to the formation of mammary tumors. Our HYPOTHESIS is that overexpression of Wip1 contributes to human breast cancer development and drug resistance. To test this, three SPECIFIC AIMS are proposed: 1. To determine if overexpression of Wip1 results in the formation of mammary tumors in vivo. 2. To test if reducing Wip1 expression can reduce the tumorigenicity and increase the sensitivity to therapeutics of human breast cancer cell lines that overexpress Wip1. 3. To assess if knockdown Wip1 expression will translate into a therapy in an orthotopic human breast xenograft model. STUDY DESIGN: In order to determine if Wip1 overexpression can result in the formation of mammary tumors in vivo, we will generate transgenic mice expressing Wip1 in the mammary epithelium under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). To test if reducing Wip1 expression can reduce tumorigenicity and enhance the sensitivity to therapeutics, we will use a small interfering RNA (siRNA) approach to generate derivatives of Wip1-overexpressing human breast cancer cell lines that express reduced levels of Wip1. The tumorigenicity and sensitivity to therapeutics of each parental cell line and its derivatives will then be compared. To test if we could translate this as a therapy to treat breast cancer, orthotopic human breast xenografts in nude mice will be treated with chemotherapy alone or chemotherapy plus Wip1 siRNA by a systemic delivery. POTENTIAL OUTCOMES AND BENEFITS OF THE RESEARCH: The proposed study will determine whether overexpression of Wip1 results in the formation of mammary tumors in vivo and whether reducing Wip1 level decreases the tumorigenicity and/or increases the sensitivity to therapeuti

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BACKGROUND:

Breast cancer is the most common cancer among women in the western world. According to American Cancer Society's estimation, approximately 211,300 new invasive cases of breast cancer will be diagnosed among women in the United States, and nearly 55,700 additional new cases of *in situ* breast cancer are expected to occur among women during 2003. Despite improvement in diagnosis and treatment of cancer, approximately a quarter of breast cancer patients will die of their disease. It is therefore very important to develop and apply of novel, mechanism-based therapies for the treatment of human breast cancer. The key to the development of such rational therapeutic approaches lies in the identification of genes and biochemical pathways that involved in the development of breast cancer (1).

Overexpression of a wild-type p53-inducible serine-threonine phosphotase Wip1 (also know as PPM1D), through gene amplification has recently been found in a variety of human cancers, including neuroblastomas (28%), breast (16%) and ovarian (25%) cancers (2-6). The Wip1 gene is located in the 17q23 amplicon, a novel region of amplification that has been linked with poor prognosis of breast cancer patients (7, 8). The protein encoded by Wip1 gene is a member of the type 2C protein phosphatase (PP2C) family of Ser/Thr protein phosphatases. PP2C family members are known to be negative regulators of cell stress response pathways. The Wip1 protein localizes to the nucleus and contains three distinct domains: an amino-terminal domain, a PP2C functional domain, and a carboxyl-terminal domain. Analysis of Wip1 revealed potential binding sites for transcription factors including NF-κB, E2F1, and c-Jun, all these three transcription factors are also reported containing oncogenic properties (7). Although expression of Wip1 is induced by p53, it negatively regulates p53 functional activity (9, 10). The oncogenic properties of overexpressed Wip1 on cells in culture include: (1) attenuation of the apoptotic process induced by serum starvation; and (2) transformation of wild-type p53-containing cells in cooperation with Ras or Her-2/neu (c-erbB-2) (2, 3, 11). Overexpressed Wip1 can expedite tumor formation both *in vitro* and *in vivo* by inactivation of a pro-apoptotic factor, p38 mitogen-activated protein kinase, and suppression of key tumor suppressor genes, such p53, p19ARF (upstream regulator of p53), and $p16^{INK4a}$ (an inhibitor of the cyclin D-CDK4-CDK6 protein kinases, which in turn are upstream regulators of pRb tumor-suppressor protein) (3, 11). All these functions of Wip1 may contribute to cancer development. In addition, gene amplification of Wip1 was also reported to be associated with poor prognosis in patients with ovarian cancer and neuroblastoma (5, 6). Gene knockout studies shows that fibroblasts derived from Wip1 null embryos have decreased proliferation rate and enhanced p53 functional activity and elevated expression of p19ARF and $p16^{INK4a}$, i.e., deletion of Wip1 activated two distinct tumor suppressors: p53 and pRb pathways (3, 12). These data suggest that Wip1 might be involved in breast cancer development through inactivation of the key tumor suppressor pathways. However, there is no direct evidence yet to demonstrate if overexpression of Wip1 results in the formation of mammary tumors and we will test this in our **Specific Aim 1**.

Double-stranded RNAs, also known as small interfering RNA (siRNA), are recognized as a powerful tool to induce loss-of-function phenotypes by inhibiting gene expression post-transcriptionally (13). The small interfering RNA-mediated gene regulation pathway is the best characterized post-transcriptional gene silencing mechanism known as RNA interference (RNAi) (13, 14). RNAi is triggered by 21-23 nucleotide double-stranded RNA (dsRNA), which induces the formation of a ribonucleoprotein complex that mediates sequence-specific cleavage of the transcript cognate with the input dsRNA. Currently, both synthetic siRNAs as well as vector-based expression systems of functional short hairpin RNAs (shRNAs) have been used successfully to silence gene expression in a variety of biological systems, including *in vivo* delivery in adult mice (13, 15-17). Therefore, RNAi has been adapted as a functional genomics tool and it has potential as a therapeutic approach (13, 14, 18, 19). In the current proposal, we will employ this powerful tool and will test if knockdown Wip1 expression by siRNAs will affect the phenotypes of breast cancer cells with Wip1 overexpression in our **Specific Aim 2** and **Aim 3**.

Preliminary Studies and Results:

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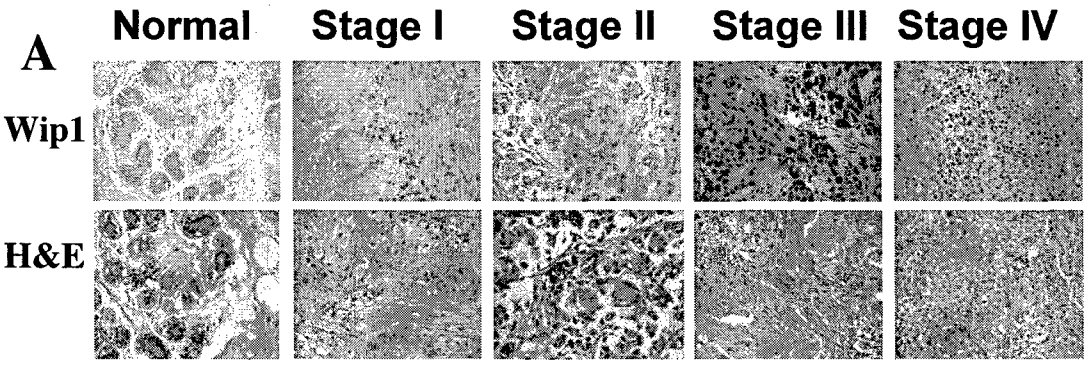
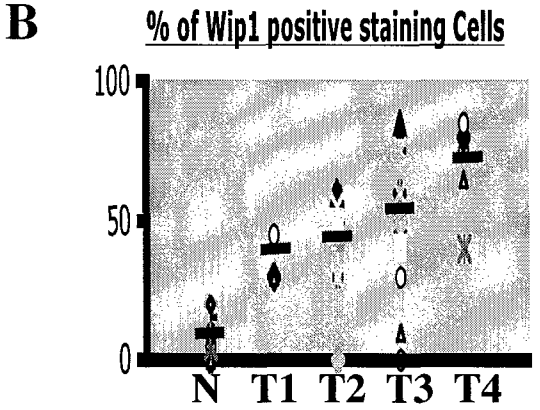
1. The levels of Wip1 protein expression correlate with the aggressiveness of human breast cancer. Although gene amplification of Wip1 has been reported in human breast cancer, it is not yet known whether the level of Wip1 protein is associated with the aggressiveness of human breast cancer. To test that, we screened Wip1 expression in normal breast (n = 10) and breast tumor tissue samples in different stages (n = 50) by immunohistochemistry method using tissue microarray slides (Figure 1). We observed most of the normal breast epithelial cells express undetectable or very low level of Wip1 protein (average positive cells < 8%). Whereas, most of the tumor cells express high level of Wip1 with average positive cells of 37% in Stage I tumors (n = 5), 40% in Stage II tumors (n = 20), 50% in Stage III tumors (n = 20), and > 69% of Stage IV tumors (n = 20). Compared with normal breast epithelial cells, expression of Wip1 is dramatically increased in breast tumor cells ($P < 0.0001$). In addition, expression of Wip1 in late stage tumors (Stage III and Stage IV, n = 25) is significantly higher than early stage tumors (Stage I and Stage II, n = 25) ($P < 0.01$). These results suggest that **the levels of Wip1 expression correlate with the aggressiveness of breast cancer and overexpression of Wip1 contributes to the tumorigenesis of human breast cancer,** we will test this in our **Specific Aim 1.**

Figure 1. Wip1 expression correlates with tumor grade of human breast cancer. We analyzed 10 normal breast tissue samples (including two normal nipple and eight normal breast tissues) and 50 cases of breast cancer at different stages in Histo-Array slides purchased from IMGENEX (San Diego, CA). Detailed information about the slide is available on-line (http://www.imgenex.com/histoarrays/). These tumors include 4 cases at stage I (T1), 21 cases at stage II (T2), 20 cases at stage III (T3), and 5 cases at stage IV (T4). (*A*). A representative image of the immunostaining of Wip1 protein expression in normal and different stages of human breast cancer. (*B*). Percentage of Wip1 positive cells in normal and different stages of breast cancer.





2. Blocking Wip1 expression induces spontaneous apoptosis and confers sensitivity to drugs. To test whether Wip1 could be a target for cancer therapy, we did a pilot study by using small interfering RNA

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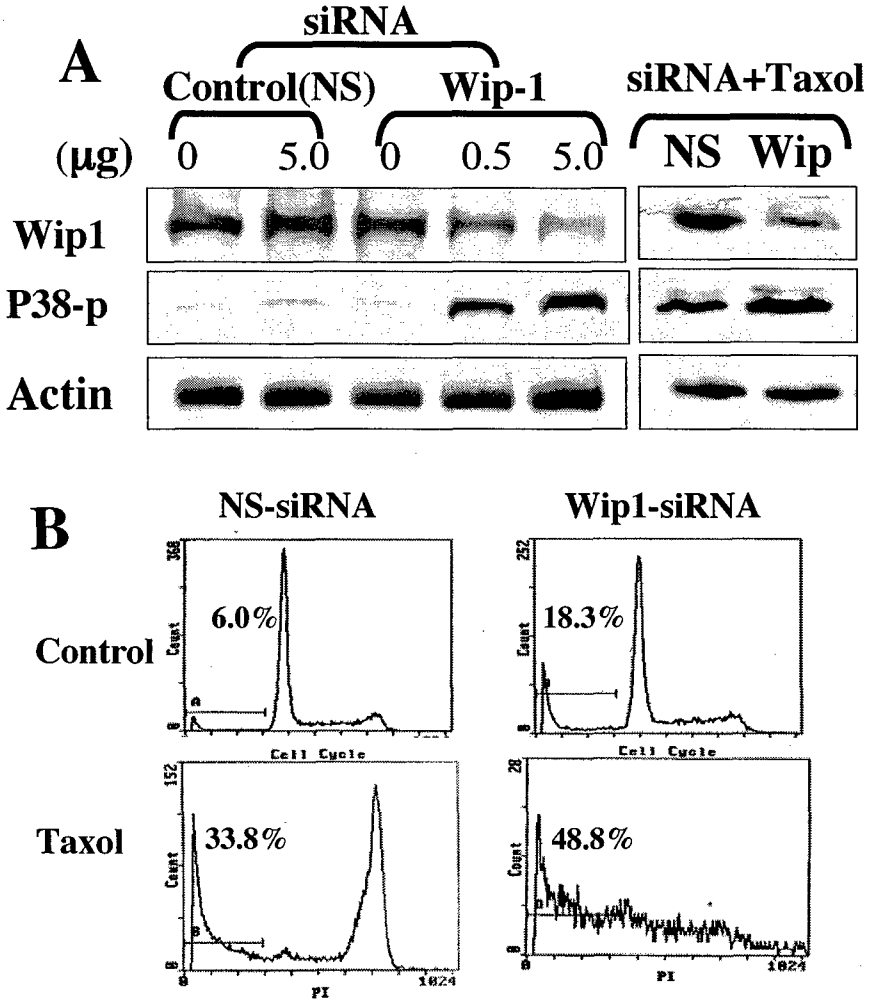
(siRNA) to block Wip1 expression, we observed that increased spontaneous apoptosis detected by FACS analysis were accompanied with reduced protein expression of Wip1 measured by western blot analysis in a dose-dependent manner in human MCF7 cells with Wip1 gene amplification and protein overexpression (Figure 2). Similar results was also reported by using antisense oligonucleotides to down-regulate Wip1 expression in Neuroblastoma (5). **This result suggests that Wip1 may be an important target for the development of anticancer therapeutics and we propose to further validate it in our Specific Aim 2.**

Figure 2. Knocking-down Wip1 expression by a specific small interfering RNA (siRNA) against Wip1 results in enhanced p38 phosphorylation (A) and increased spontaneous as well as drug-induced apoptosis (B). (*A*). A dose-dependent effect of Wip1 siRNA on Wip1 expression in breast cancer MCF-7 cells. (*B*). For Taxol treatment, cells were transfected with siRNA (5.0 μg) using SN liposome for 16 hrs and replaced with fresh medium before addition of 0.1 μM of Taxol and were incubated for another 24 hrs before harvesting. Cells with or without treatment (Control) were then washed with PBS and fixed in 75% ethanol, washed again and stained with propidium iodide (25 μg/ml), and analyzed by a fluorescence-activated cell sorter (FACS).



3. Introducing Wip1 expression in Wip1 null cells confers resistance to drugs. To test whether expression of Wip1 could confer resistance to anti-cancer drugs, we transiently transfected Wip1 into Wip1 knockout mouse embryo fibroblasts (MEF) and benign breast cancer cell HBL-100, which expression low level of Wip1.

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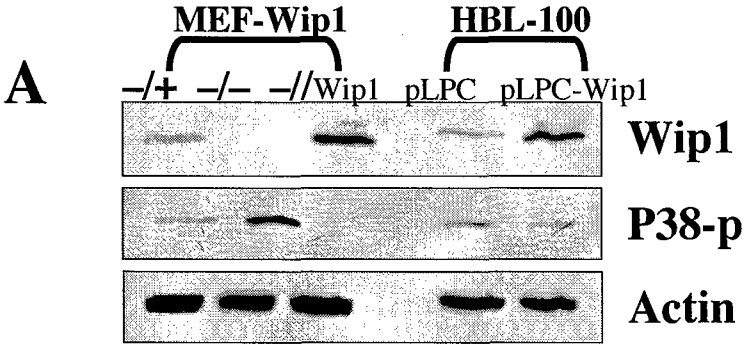
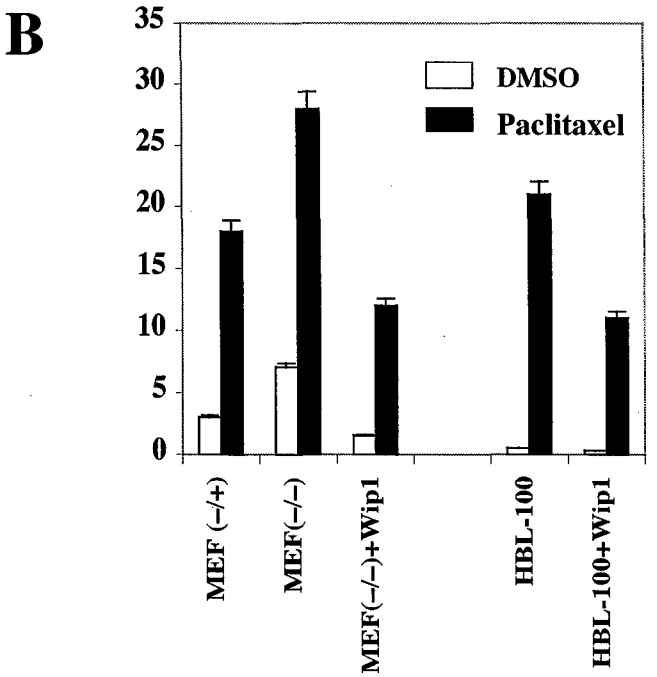
We observed that Wip1 (–/–) MEFs is more sensitive to drugs than the heterozygous Wip1 (–/+) MEFs and expression of Wip1 in either Wip1 null MEFs or HBL-100 cells confers resistance to drugs (Figure 3). **These results further suggest that expression of Wip1 may contribute to tumor cells resistance to drugs and reducing Wip1 may enhance the therapeutic efficacy of anti-cancer drugs. Therefore, we propose to test this in a systemic gene therapy setting in an orthotopic breast cancer model *in vivo* in our Specific Aim 3.**

Figure 3. Introducing Wip1 expression in Wip1 (–/–) MEFs or HBL-100 benign breast cancer cells represses p38 phosphorylation (A) and confers resistance to drugs (B). Mammalian expression plasmid of human full-length Wip1 cDNA, pLPC-Wip1, was transfected into Wip1 (–/–) MEFs (–//Wip1) with FuGENE 6 lipid (Roche Molecular Biochemicals, Indianapolis, IN) and HBL-100 cells (pLPC-Wip1) with SN liposome, respectively. Cells were grown for 36 hours and were then harvested for protein expression analysis (***A***) or further treated with paclitaxel (Taxol) or DMSO for another 24 hour. After treatment with Taxol, cells were then washed with PBS twice and fixed with 70% alcohol and stained with Hoechst 33342 (0.5 μg/ml, Sigma, St. Louis, Missouri). Events of apoptotic nuclei were counted under a fluorescence microscope and the mean values of 500 cells were plotted (***B***).





PROJECT DESCRIPTION

HYPOTHESIS/OBJECTIVE:

Based on our preliminary studies and previous reports, we hypothesized that is that overexpression of Wip1 contributes to human breast cancer development and drug resistance. The objectives of this proposed study are to determine whether reducing Wip1 expression can reduce the tumorigenicity and increase the sensitivity to therapeutics of human breast cancer cells that overexpress Wip1. Our long-term goal is to identify lead compounds that reduce the expression of Wip1 and develop new cancer treatments that reduce Wip1 overexpression.

SPECIFIC AIMS:

The long-term goal of this proposal is to develop a novel therapeutic method to reduce the mortality of breast cancer patients by targeting the Wip1 oncogene. To achieve this goal, we propose the following three specific aims to test the feasibility:

Aim1: To establish mammary-specific Wip1 transgenic mouse model for the evaluation of the pathological roles of Wip1 gene in breast tumorigenesis and/or breast development. By screening the expression of Wip1 in human breast cancer tissue samples, we demonstrated that the level of Wip1 expression correlates with tumor stages of human breast cancer. We will establish a mammary-specific Wip1 transgenic mouse model to test if overexpression of Wip1 contributes to the development of mammary tumor by MMTV-LTR mediated expression of Wip1 in the mammary epithelium.

Aim2: To validate Wip1 as a potential therapeutic target for breast cancer *in vitro* and *in vivo* by a retroviral vector-mediated expression of Wip1 shRNA. By transient transfection of Wip1 siRNA, we have observed that knowdown Wip1 expression results in increased spontaneous apoptosis and sensitization to drugs. We will further test if we could obtain a Wip1 protein level dependent effect on cell growth, apoptosis, and tumorigenesis in stable cells express Wip1 shRNA *in vitro* and *in vivo*.

Aim3: To assess if knockdown Wip1 expression will sensitize breast cancer cells to chemotherapy and enhance animal survival rate in an orthotopic human breast xenograft model. Results obtained in Specific Aim 1 and Aim 2 will validate if Wip1 is a therapeutic target of breast cancer, we will then test if this could translate into a therapy in an orthotopic model by a systemic delivery of Wip1 siRNA. This is particularly important for therapy of breast cancer as breast cancer is a metastatic disease and systemic delivery (through i.v. injection) is required. Success of this proposal will provide proof of concept for a therapy targeting on Wip1 gene in breast cancer and may lead to a further clinical trial to develop a novel therapy for breast cancer.

STUDY DESIGN:

Aim1. To establish mammary-specific Wip1 transgenic mouse model for the evaluation of the pathological roles of Wip1 gene in breast tumorigenesis and/or breast development.

Rationale: Gene amplification and overexpression of Wip1 have been detected in human breast cancer. Our preliminary data further linked the levels of Wip1 expression to the aggressiveness of breast cancer, i.e., the higher the Wip1 expression, the higher the tumor grade. By far, there is no direct evidence or model system to test if overexpression of Wip1 results in the formation of mammary tumors. Therefore, we proposed to establish a mammary-specific Wip1 transgenic mouse model for the evaluation of the pathophysiological roles of Wip1 gene in breast tumorigenesis and /or breast development.

Approach: We have constructed an expression cassette for the generation of mammary-specific expression of Wip1 transgene by subcloning the 1.8 kb full-length *Bam HI* fragment of wild-type human Wip1 cDNA into the

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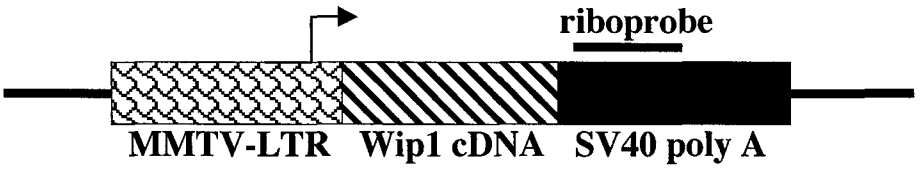
Bam HI site of plasmid p206 downstream of the MMTV-LTR promoter/enhancer, in order to drive expression in the mammary epithelium. The polyadenylation signal of the SV40 (SV40 poly A) was induced to ensure efficient processing of the RNA transcript. To confirm the MMTV/Wip1 transgene expression in the founder cell lines of transgenic mice, a ribonuclease protection ananlysis of the total mammary gland RNA will be performed by using a riboprobe generated against the transgene-specific SV40 sequence, as shown in Figure 4. The expression cassette will be prepared and injected into one cell zygotes of FVB/N mice following the procedures of our institution's Genetically Engineered Mouse Core Facility and as described (20, 21). To identify transgenic animals, genomic DNA will be isolated from 0.5 cm clippings of mouse tail, and PCR amplified using a Wip1-specific forward primer (CTGGGAGTGAGCGTCTTC) and an SV40-specific reverse primer (TATGTCACACCACAGAAG), to generate a transgene-specific amplification product. Once the MMTV-Wip1 transgenic mouse is available, organ and tissue distribution of Wip1 cDNA and protein will be analyzed in the transgenic mice. Following the recommendations from the Annapolis meeting of the Workshop on Mouse Models of Human Breast Cancer (22, 23), we will systemically collect the whole mount and histological samples from the transgenic mice and age and parity matched wild type controls during development (4, 8, 12, and 14 weeks after birth) and during the cycle of pregnancy, lactation, and involution cycles. A pathologist collaborator will responsible for the histological analysis of the transgenic mouse tissue samples. If there are no obvious gross abnormalities in MMTV-Wip1 transgenic mouse, we will concentrate on mammary regions where Wip1 is most abundantly expressed to search for more subtle defects. In addition, we will conduct chemical- or radiation-mediated carcinogenesis to see whether Wip1 will facilitate tumor formation under these conditions, since Wip1 expression is stress inducible (9, 10). Of course, Wip1 may be involved in more than one of the functions discussed above, and the course of our analysis will be determined by the phenotypes we observed.

Figure 4. Expression cassette used for the generation of MMTV-LTR/Wip1 mice.



Aim 2. To validate Wip1 as a potential therapeutic target for cancers *in vitro* and *in vivo* by a retroviral vector-mediated expression of Wip1 shRNA.

Rationale: Wip1 represses both p38 and p53 functional activation, while both p38 and p53 activities have been documented to be required for anti-cancer drug-induced apoptosis (24-26). We reasoned that overexpression of Wip1 may contribute to tumor cell resistance to drugs and downregulation of Wip1 may sensitize tumor cells to drug-induced apoptosis. Our preliminary data suggested that the levels of Wip1 expression correlated with patient tumor stages and introducing Wip1 expression in Wip1 null MEFs or HBL-100 breast cancer cells conferred resistance to drugs while knockdown Wip1 expression by a specific siRNA against Wip1 in Wip1–overexpressing MCF-7 cells induced spontaneous apoptosis and enhanced sensitivity of cancer cells to anti-cancer drugs. Therefore, we propose to further validate if Wip1 is a therapeutic target. The validation of a drug target involves demonstrating the relevance of the target protein in a disease process and ideally requires both gain and loss of function studies. This is accomplished primarily with highly specialized knock-out (loss of function) and knock-in (gain of function) animal models that are capable of mimicking the targeted disease states, or with the use of small molecules (inhibitors, agonists, antagonists), antisense nucleic acid constructs, ribozymes and neutralizing antibodies. To validate whether Wip1 could be a target for anticancer treatment, we will use a small interfering RNA approach to generate derivatives of Wip1-overexpressing human breast cancer cell lines that express reduced levels of Wip1 because of the target-specificity and efficiency of RNA interference in silencing endogenous target gene expression (loss of function analysis) (13, 14, 18, 19). The

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MCF7 (p53+, ER+), BT474 (p53+, ER+), and MDA-MB-361 (p53-, ER-) human breast cancer cell lines express high levels of Wip1. BT474 and MDA-MB-361 also express very high level of HER-2/neu with HER-2/neu amplification, whereas MCF7 express moderate level of wild type HER-2/neu. These cell lines therefore represent breast cancers arisen through different molecular mechanisms. We will generate stable derivatives of these cell lines that express reduced levels of Wip1.

Approach: We have constructed three different Wip1 short hairpin RNA (shRNA) constructs in pSUPER.retro.puro plasmid vector (OligoEngine, Seattle, WA). These Wip1 shRNA constructs were designed to target two Wip1 coding region and one 5' non-translated region (5'-NTR), respectively. We will infect MCF-7, BT474, and MDA-MB-361 cells with these pSUPER-Wip1 shRNA viral supernatant and the expression of Wip1 protein in the parental and each derivative will be monitored. The tumorigenicity and sensitivity to therapeutics of each parental cell line and its derivatives will then be compared in cell culture *in vitro* and in animal model *in vivo*. For the *in vitro* experiments, the Wip1 derivatives and their parental cells will treat with different anti-cancer drugs that are used clinically to treat human breast cancer, such paclitaxel, cis-platin, etc, we will measure the relative expression of Wip1, phospho-p38, p53, and a few apoptotic markers, such as activation of caspase enzymes, bax and cytochrome c translocation, bcl-2 phosphorylation, etc. We will use MTT, soft agar assay, flow cytometry to measure cell proliferation, apoptosis and anchorage-independent cell growth of each Wip1 derivatives and the parental cells. For the *in vivo* experiments, we will inoculate these Wip1 derivatives and their parental cells into mouse mammary fat pad, the tumor formation, growth rate and animal survival rate will be monitored and recorded. If we did observe that knockdown Wip1 expression enhances cells' sensitivity to drugs, we will further test if transfection of Wip1 cDNA into stable cell lines derived from one of the Wip1 shRNA construct that target specifically the 5'-NTR (which is not suppose to inhibit Wip1 cDNA-mediated transcription/translation of Wip1 protein) results in reversion of the phenotype (gain of function analysis). We expect to see that reduce Wip1 expression will reduce cell growth rate both *in vitro* and *in vivo* while reintroduction Wip1 in the 5'-NTR shRNA generated stable cells will reverse the phenotype.

Aim 3. To assess if knockdown Wip1 expression will sensitize breast cancer cells to chemotherapy and enhance animal survival rate in an orthotopic human breast xenograft model by a liposome-mediated systemic delivery of Wip1 siRNA.

Rationale: The studies proposed in Aim 2 are not a therapy. However, if we could obtain a Wip1 protein level dependent effect on cell growth, apoptosis, and tumorigenesis in Aim 1 and Aim 2, we will then test if target Wip1 could be translate into a potential therapy for breast cancer in pre-clinical gene therapy model derived from human breast cancer cell lines. To avoid any non-specific effect generated from the using of retroviral vector in Aim 2, we will use chemically synthesized double-stranded siRNA oligos for the *in vivo* therapeutic analysis of the combination effect between Wip1 siRNA and anti-cancer drugs.

Approach: To test this, an orthotopic human breast xenograft model in nude mice will be established as described previously (27). Briefly, cells (MCF-7, BT474, MDA-MB-361) for in vivo experiments were grown to 70% confluence. Cells were then trypsinized, washed with 10% serum, centrifuged and re-suspended in Hanks' balanced salt solution at $1x10^6$cells/50μl for injection into the mammary fat pad of mouse with a 30-gauge needle after anesthesia with pentobarbital (50 mg/kg). We will use our recently developed SN-liposome to deliver specific or non-specific double-stranded siRNA (27, 28). Tumor-bearing mice will be divided into six groups (10 mice per group) for each cell line and treated with: (1) PBS, (2) 21-nucleotide (nt) double-stranded Wip1 specific siRNA/SN-liposome, (3) 21-nt non-specific double stranded siRNA/SN-liposome, (4). paclitaxel, at 10mg/kg/dose, (5). paclitaxel/Wip1 siRNA/SN-liposome, and (6) paclitaxel/non-specific siRNA/SN-liposome, by intraperitoneal (i.p.) injection of paclitaxel once a week and i.v. injection of siRNA/SN-liposome at twice per week. The tumor volume and survival rate will be monitored. At autopsy, the tumor samples will be analyzed for Wip1 expression by Western blot and immunohistochemistry, and apoptosis by in situ TUNEL assay. We expect to see that a combination of Taxol with Wip1 siRNA will yield a dramatic therapeutic effect

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than that of the other combinations or any single agent. Here, we proposed to use paclitaxel for a combination study, if our Aim 2 shows that cis-platin or any other drugs could yield a better synergistic effect than paclitaxel, then we will switch to cis-platin or other agent for this *in vivo* therapy. In addition, if the MMTV/Wip1 trangenic mice developed mammary tumors, we will then use this model system to further validate the therapeutic efficacy of Wip1 siRNA with or without combination with ant-cancer drugs.

POTENTIAL OUTCOMES AND RELEVANCE:

It is not yet know whether Wip1 is a therapeutic target for breast cancer, even though genetic studies suggested that overexpression of Wip1 resulting from gene amplification contributes to the development of human breast cancer. The proposed study will directly investigate whether reducing Wip1 level decreases the tumorigenicity and/or increases the sensitivity to therapeutics of cancer cell lines that overexpress Wip1. The siRNA used in the proposed study to modulate Wip1 expression can also be utilized as a therapeutic agent to target Wip1. Thus, the finding of this study could not only lead to better understanding of how breast cancer develops, but also translate to more effective treatments for human breast cancer.

TIMELINE:

Year 1 **Year 2**

Establish MMTV-LTR/Wip1 transgenic mice
Phenotype analysis of MMTV-Wip1 trangenic mice
Tissue distribution of Wip1 transgene expression
Mammary tumor formation
Generate derivatives of reduced Wip1 expressing stable cell lines
Test these derivatives for drug sensitivity *in vitro* and tumor formation *in vivo*
Test systemic delivery of Wip1 siRNA with or without combination with drugs *in vivo*

DISSEMINATION PLAN:

The data shown in the preliminary results section will be presented in the annual meeting of American Association for Cancer Research (2005). With a few minor experiments, we will put these results together as a manuscript and submit to Cancer Research. The phenotype analysis of mammary-specific Wip1 transgenic mice and Wip1 siRNA on cell growth, proliferation, and tumor formation will be put together as a manuscript and submit to Nature Medicine or Nature Genetics. The therapeutic effect of systemic delivery of Wip1 siRNA with or without combination with drugs will put together as a manuscript and submit to Cancer Research or Gene Therapy. All the results will be presented in the major national meetings such as the annual meetings of American Association for Cancer Research or American Society for Gene Therapy.

PROJECT DESCRIPTION

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10. Takekawa, M., Adachi, M., Nakahata, A., Nakayama, I., Itoh, F., Tsukuda, H., Taya, Y., Imai, K. (2000), p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. EMBO J., 19(23): 6517-6526.
11. Bernards, R. (2004), Wip-ing out cancer. Nat Genet, 36(4): 319-320.
12. Choi, J., , Nannenga, B., Demidov, O.N., Bulavin, D.V., Cooney, A., Brayton, C., Zhang, Y., Mbawuike, I.N., Bradley, A., Appella, E., Donehower, L.A. (2002), Mice deficient for the wild-type p53-induced phosphatase gene (Wip1) exhibit defects in reproductive organs, immune function, and cell cycle control. Mol. Cell. Biol., 22(4): 1094-1105.
13. Caplen, N. J. (2004), Gene Therapy Progress and Prospects. Downregulating gene expression: the impact of RNA interference. Gene Ther., 11(16): 1241-1248.
14. Zamore, P. D. (2001), RNA interference: Listening to the sound of silence. Nat Med, 8(9): 746-750.
15. Xia, H., Mao, Q., Paulson, H.L., Davidson, B.L. (2002), siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol, 20(10): 1006-1010.
16. McCaffrey, A. P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J., Kay, M.A. (2002), RNA interference in adult mice. Nature, 418(6893): 38-39.
17. McCaffrey, A. P., Nakai, H., Pangey, K., Huang, Z., Salazar, F.H., Xu, H., Wieland, S.F., Marion, P.L., Kay, M.A. (2003), Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol, 21(6): 639-644.
18. Devroe, E., Silver, P.A. (2004), Therapeutic potential of retroviral RNAi vectors. Expert Opin Biol Ther, 4(3): 319-327.
19. Mittal, V. (2004), Improving the efficiency of RNA interference in mammals. Nat Rev Genet, 5(5): 355-365.
20. Webster, M. A., Hutchinson, J.N., Rauh, M.J., Muthuswamy, S.K., Anton, M., Tortorice, C.G., Cardiff, R.D., Graham, F.L., Hassell, J.A., Muller, W.J. (1998), Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. Mol Cell Biol, 18(4): 2344-2359.
21. White, D. E., Cardiff, R.D., Dedhar, S., Muller, W.J. (2001), Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene, 20(48): 7064-7072.

PROJECT DESCRIPTION

22. Cardiff, R. (2001), Validity of mouse mammary tumor models for human breast cancer: comparative pathology. Microsc Res Tech, 52(2): 224-230.
23. Cardiff, R. D., Anver, M.R., Gusterson, B.A., Hennighausen, L., Jensen, R.A., Merino, M.J., Rehm, S., Russo, J., Tavassoli, F.A., Wakefield, L.M., Ward, J.M., Green, J.E. (2000), The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. Oncogene, 19(8): 968-988.
24. Liao, Y., Hung, M.C. (2003), Regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenoviral protein E1A-mediated sensitization to apoptosis. Mol Cell Biol, 23(19): 6836-6848.
25. Brown, J. M., Wouters, B.G. (1999), Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res., 59 (1391-1399.
26. El-Deiry, W. S. (2003), The role of p53 in chemosensitivity and radiosensitivity. Oncogene, 22(47): 7486-7495.
27. Liao, Y., Zou Y.Y., Xia, W.Y., Hung, M.C. (2004), Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by a non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer. Cancer Gene Ther., 11(7): 1-9.
28. Zou, Y., Peng, H, Zhou, B, Wen, Y, Wang, SC, Tsai, EM, Hung, MC. (2002), Systemic tumor suppression by the preapoptotic gene bik. Cancer Research, 62(1): 8-12.

Liao, Y | BCTR0504146

P.I. Last Name, First Initial | **Tracking ID Number**

MASTER APPLICATION PACKET BUDGET FORM

Budget Category	**Detailed Budget for Entire Budget Period**			
ITEMIZE ALL CATEGORIES IN BUDGET JUSTIFICATION	**Year One**	**Year Two**	**Year Three**	**Dollar Amount Requested (U.S. currency, omit cents) TOTAL**
Personnel Costs* (Itemize in Budget Justification)	74,500.00	78,500.00		153,000.00
Supplies (Itemize in Budget Justification)	20,000.00	20,000.00		40,000.00
Equipment (Must not exceed more than 30% of requested direct costs)				0.00
Patient Care Costs (Itemize in Budget Justification)				0.00
Travel (Itemize in Budget Justification)	1,500.00	1,500.00		3,000.00
Other Expenses (Itemize in Budget Justification)	2,000.00	2,000.00		4,000.00
Consortium/Contractual Costs: (Detailed budget must be attached)				0.00
Subtotal Direct Costs:	98,000.00	102,000.00	0.00	200,000.00
Indirect cost allocation (Must not exceed 25% of requested direct costs)	25,000.00	25,000.00		50,000.00
Total Funding Request (Cannot exceed $250,000 for all combined years)	123,000.00	127,000.00	0.00	250,000.00

***P.I. and KEY personnel must have a percent of effort, even if not being compensated, and must be included in the Budget Justification. Maximum annual base salary is $125,900. Must include Fringe Benefit rate in Budget Justification. Please use this form and copy for any additional required budgets.**

MASTER APPLICATION BUDGET JUSTIFICATION
DO NOT EXCEED TWO PAGES.

PERSONNEL:
Yong Liao, Ph.D., P.I. (70% effort), will be responsible for the project and will supervise the proposed laboratory experiments. In addition, the P.I. will evaluate the results of experiments and prepare the relevant data for presentation and publication. The P.I. will also prepare the reports to the Komen Foundation. Salary requested: 1st year, 70% x $53,414,(base salary) +$8,439 (benefits) = $45,828; 2nd year, 70% x $56,618,+ $ 8,761 = $48,394.4 (total: $94,222.4).

Mien-Chie Hung, Ph.D., Collaborator, (0% effort) will supervise and assist conduction of all the experiments proposed in this project. Dr. Hung has over 15 years of experience in supervising and administering research programs in the field of cancer research.

To be named, B.S., Research Assistant, (100% effort) will assist the PI with most of the experiments proposed in this project. Salary reqested: 1st year, $22,400 (base salary) +$6,272 (benefits) = $28,672; 2nd year, $23,520 + $ 6,586.5 = $30,105.6 (total: $58,777.6).

Yun Wu, M.D., Ph.D., Collaborator, (10% effort) will responsible for histological analysis of transgenic mice. Salary requested: none.

Fringe Benefits is based on the institutional policy at 28% of the requested salary.

SUPPLIES:
Phospholipids will be used for liposome formulations

Peptide/Peptide-lipid Synthesis: for targeted liposome formulations.

Pathology: for apoptosis, toxicity, histological, and tumor targeting analysis

Chemicals including common chemicals, reagents, molecular biology kits for purification of RNA, DNA and proteins.

Plasmid kits: for plasmid purification

Mice: for tumor model, GEM facility services, and all animal experiments

Tissue Culture Supplies includes, disposable sterile dishes, flasks, multiwell plates, pipets, cryotubes for freezing materials, etc. for all in vitro studies including the optimization of gene expression system and targeted formulations.

TRAVEL:
Funds are requested for Principal Investigator and key personnel to travel to one scientific meeting per year.

OTHER EXPENSES:
Publication and Report Cost are for the preparation of graphs, charts, slides, and photographs of manuscripts for presentation at scientific meetings.

Animal Maintenance: Nude mice have to be kept in the nude mice housing area with laminar flow and SPF barrier. We keep no more than 5 mice in one cage and some mice will be kept for longer than one year (e.g. survival assay).

Electronic and Glass Shop Fees: Fee charged for the upkeep and maintenance of laboratory equipment.

Liao, Y | BCTR0504146

P.I. Last Name, First Initial | **Tracking ID Number**

OTHER SOURCES OF FINANCIAL SUPPORT

Required in Master Application Packet only.

The Komen Foundation does not allow for overlap funding. Potential conflicts or overlaps in funding must be identified.

Is support for this project being sought somewhere else? (Please check one.) ☐ Yes ☑ No
If yes, list the agencies, titles of the project(s), total funding requested, and specify areas where there are overlaps in budget requests.

In addition, list ALL sources of support (pending or current) including federal, nonprofit, industry or other support. Give complete titles of all grants, as well as total award, inclusive funding period, role of the P.I., and percent of time devoted to each grant. (Attach additional sheets if necessary.)

DAMD17-01-1-0300, as PI (100% effort)
USAMRMC DOD,
Total award: $150,000.00
Funding date: June 1, 2001 to May 30, 2004

Title: "Tumor Suppression and Sensitization to Taxol–Induced Apoptosis of E1A in Breast Cancer Cells"

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its antitumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy.

BIOGRAPHICAL INFORMATION

Information should be submitted for the Principal Investigator and key personnel. Please use a separate form for each person.

NAME LIAO Yong TITLE Instructor

EDUCATION *(Begin with baccalaureate or initial professional education, such as nursing, include postdoctoral training)*

INSTITUTION and LOCATION	DEGREE	YEAR(S) CONFERRED (Note Qualifications in RFA)	FIELD OF STUDY
North Sichuan Med. College, Sichuan,China	Undergraduate	1981-1984	Medicine
Xinjiang Medical College, Xinjiang, China	M.Sc.	1989-1992	Medicine
Shanghai Med. University, Shanghai, China	Ph.D.	1992-1995	Oncology
The Univ. of Texas MD Anderson Cancer Center, Houston, Texas	Post-doc	1997-2002	Molecular Oncology

PROFESSIONAL EXPERIENCE: Please list, in chronological order, concluding with present position, previous employment, experience and honors. List, in chronological order, the titles, authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. **DO NOT EXCEED TWO PAGES.**

WORKING EXPERIENCE:

1984.7—1989.8 Surgical resident, Department of General Surgery, Zhong Jiang County Hospital, Sichuan, P.R. China

1995.5–1996.12 Researcher, Liver Cancer Institute, Zhong Shan Hospital, Shanghai Medical Univeristy, Shanghai, P. R. China

2002.9–present Instructor, Department of Molecular & Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA

HONORS & AWARDS:

1993: Winner of "Guang Hua Scholarship" due to excellent credits
1994: Excellent Graduate Student recognized by the Graduate School, Shanghai Medical University
1995: Seeding Grant for Gene Therapy, Awarded by Shanghai Medical University
1996: 2nd Prize of Achievements in Liver Cancer Research awarded by the Funds of Zhao-You Tang &. Shanghai Construction Bank, Shanghai, P.R. CHINA
1995: 1st Prize of Science &. Technology Progress of Xinjing Autonomous Government, Xinjing, P.R CHINA
1995: 2nd Prize of Science &. Technology Progress Awards of the Ministry of Health, P.R CHINA 1999: Post-doctoral Fellowship, awarded by the M.D. Anderson Breast Cancer Program/U.S. Army Breast Cancer Research Training Grant.
1996: Grand Prize of "8.5" National Major Achievements of Science &. Technology Awarded by National Science &. Technology Committee, P.R..China
1996: First Prize of Science &. Technology Progress Awards of Shanghai, Shanghai Municipality, Shanghai, P.R. CHINA
1999: Post-doctoral Fellowship, awarded by the M.D. Anderson Breast Cancer Program/U.S. Army BCRT program
2001: Postdoctoral Fellowship award, U.S. Department of the Army Breast Cancer Research Program
2003: 2nd Prize of Science &. Technology Progress Awards of Shanghai , Shanghai Municipality, P.R. China
MEMBERSHIP:

Associate member, American Association of Cancer Research (AACR)
Member, American Association for the Advancement of Science (AAAS)
Active Member, American Society of Gene Therapy (ASGT)
Life-time Member, The Society of Chinese Bioscientists in America (SCBA)

PUBLICATIONS:

• Liao Y, Hung MC: A new role of protein phosphotase 2A in adenoviral E1A protein mediated sensitization to anti-cancer drug–induced apoptosis in human breast cancer cells. Cancer Res September 1, 2004; 64: In press.
• Xia WY, Chen JS, Zhou X, Sun PR, Lee DF, Liao Y, Zhou BH, Hung MC: Phosphorylation/cytoplasmic localization of p21 CIP1/WAF1 is associated with HER2/neu overexpression and provides a novel combination predictor for poor prognosis in breast cancer patients. Clin. Cancer Res. 2004; 10(11):3815-24.
• Liao Y, Zou YY, Xia WY, and Hung MC: Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by a non-viral mediated systemic delivery of E1A gene in orthotopic xenograft human breast cancer. Cancer Gene Therapy, 2004; 11 (7): 1-9.
• Liao Y, Hung MC: "The regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenovrial protein E1A-mediated sensitization to apoptosis." Molecular & Cellular Oncology, 2003; 23(19): 6836-6848, 2003.
• Deng J, Zhang H, Kloosterbore F, Liao Y, Klostergaard J, Levitt ML and Hung MC: Ceramide is not a second messenger for ultraviolet-induced apoptosis: Evidences from E1A transfectant system. Oncogene, 2002; 21(1): 44-52 (2002).
• Liao Y*, Zhou BP*, Xia WY, Zou YY, Spohn B, Hung MC: HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nature Cell Biol. 2001; 3(11): 972-982 (*equal contribution)
• Liao Y, Zou YY, Xia WY, and Hung MC: Chemosensitization by adenovirus E1A through modulation of apoptotic signalings. Clin. Cancer Res. 2001; 7(11):3777s (suppl.)
• Zhou BP, Liao Y, Xia WY, Spohn B, Lee MH, Hung MC: Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nature Cell Biol. 2001; 3(3): 245-252
• Liao Y*, Funda M*, Lee WP, Pollock RE, Hung MC: Adenovirus 5 early region 1A does not induce expression of the Ewing scarcoma fusion product EWS-FLI1 in breast and ovarian cancer cell lines. Clin. Cancer Res. 2000; 6 (10):3832-36
• Liao Y, Tang ZY, Ye SL, Liu KD, Sun FX, Huang Z: "Modulation of apoptosis, tumorigenecity, and metastatic potential with antisense H-ras oligodeoxynucleotides in a high metastatic tumor model of hepatoma: LCI-D20." J. of HepatoGastroenterol. 2000; 47:365-370.
• Lee WP, Liao Y, Robinson D, Kung HJ, Liu ET, Hung MC: Downregulation of Axl by E1A is involved in E1A-mediated cell growth suppression and pro-apoptotic activity. Mol. Cell Biology. 1999; 19(12): 8075-8082
• Tang ZY, Qin LX, Wang XM, Zhou G, Liao Y, Weng Y, Jiang XP, Lin ZY, Liu KD, Ye SL: Alterations of oncogenes, tumor suppressor genes and growth factors in hepatocellular carcinoma: with relation to tumor size and invasiveness. Chin. Med. J. 1998; 111 (4): 313-318
• Liao Y, Tang ZY, Liu KD, Ye SL, He B, Huang Z: Apoptosis of human BEL-7402 hepatocellular carcinoma cells released by antisense H-ras DNA-in vitro and in vivo studies.J Cancer Res Clin Oncol,1997;123(1):42-48
• Liao Y, Tang ZY, Liu KD, Ye SL, Li J, Huang Z, Wang DB, Segal DM: Preparation and application of anti-HBx/ anti-CD3 bispesific monoclonal antibody (BsAb) retargeting effector cells for lysis of human xenografts in nude mice. Oncol Reports,1996;3:637-644.
• Liao Y, Tang ZY, Sun FX, Ye SL, Liu KD, Huang Z: The effect of antisense H-ras on growth and metastasis of a high metastatic tumor model of human hepatoma in nude mice LCI-D20.Natl Med J China, 1996; 76(9) :650-653.

Book Chapter:

• Liao Y: Antisense Therapeutics and Application in Liver Cancer. In: Advances in Basic and Clinical Aspects of Liver Cancer Metastasis and Treatment (First Edition), Ed: Tang ZY, Shanghai Science & Technology Press, Shanghai, 2003. P:205-248
• Liao Y, Tang ZY: Apoptosis and Cancer. In: Primary Liver Cancer, (2nd Edition), Ed: Tang ZY, Shanghai Science & Technology Press, Shanghai, 1998. P:504-512

BIOGRAPHICAL INFORMATION

Information should be submitted for the Principal Investigator and key personnel. Please use a separate form for each person.

NAME Wu, Yun TITLE Assistant Professor

EDUCATION *(Begin with baccalaureate or initial professional education, such as nursing, include postdoctoral training)*

INSTITUTION and LOCATION	DEGREE	YEAR(S) CONFERRED (Note Qualifications in RFA)	FIELD OF STUDY
Shanghai Medical University, Shanghai, China	M.D.	1985-1991	Medicine
The University of Texas, GSBS/MD Anderson Cancer Center-Houston, Texas	Ph.D.	1994-1996/1998-99	Molecular Pathology

PROFESSIONAL EXPERIENCE: Please list, in chronological order, concluding with present position, previous employment, experience and honors. List, in chronological order, the titles, authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. **DO NOT EXCEED TWO PAGES.**

POST-DOCTORAL TRAINING:

08/1991 - 10/1992 Anatomic Pathology Residency, Shanghai Medical University, Shanghai, China
07-1999 - 06/2003 Anatomic and Clinical Pathology Residency, The University of Texas, Southwestern Medical Center at Dallas, Texas
07/2003 - 06/2004 Oncologic Pathology Fellowship, Memorial Sloan-Kettering Cancer Center, New York, New York

PROFESSIONAL EXPERIENCE:

08/1991 - 10/1992 Instructor, Department of Pathology, Shanghai Medical University, Shanghai, China
08/1996 - 05/1998 Project Investigator, Department of Molecular Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas
07/2004 - present Assistant professor, Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

HONORS & AWARDS:

2000 Alfred J. Knudson, Jr. Outstanding Dissertation Award

1998-1999 Pre-doctoral Training Fellow Funded by the National Institute of Health

PUBLICATIONS:

Hawk N, Sun T, Xie S, Wang Y, Wu Y, Liu J, Arlinghaus RB: Inhibition of the Bcr-Abl oncoprotein by Bcr requires phosphoserine 354. Cancer research, 62(2): 386-90, 2002
Abe JI, Che W, Yoshizumi M, Huang Q, Glassman M, Ohta S, Wu Y, Arlinghaus RB, Berk BC: Bcr in vascular smooth muscle cells involvement of Ras and Raf-1 activation by Bcr. Annals of the New York Academy of Sciences, 947:341-3, 2001
Che W, Abe J, Yoshizumi M, Huang Q, Glassman M, Ohta S, melaragno MG, Poppa V, Yan C, lerner-Marmarosh N, Zhang C, Wu Y, Arlinghaus RB, Berk BC: p160 bcr mediates platelet-derived growth factor activation of extracellular signal-regulated kinase in vascular smooth muscle cells. Circulation, 104 (12): 1399-406, 2001
Wang Y, Liu J, Wu Y, Luo W, Lin SH, Lin H, Hawk N, Sun T, Guo JQ, Estrov Z, Talpaz M, Champlin R, Arlinghaus RB: Expression of a truncated first exon BCR sequence in chronic myelogenous leukemia cells blocks cell growth and induces cell death. Cancer Research, 61 (1):138-44, 2001

BIOGRAPHICAL INFORMATION

Information should be submitted for the Principal Investigator and key personnel. Please use a separate form for each person.

NAME HUNG Mien-Chie TITLE Professor

EDUCATION *(Begin with baccalaureate or initial professional education, such as nursing, include postdoctoral training)*

INSTITUTION and LOCATION	DEGREE	YEAR(S) CONFERRED (Note Qualifications in RFA)	FIELD OF STUDY
National Taiwan University, Taiwan, ROC	B.S.	1973	Chemistry
National Taiwan University, Taiwan, ROC	M.S.	1977	Biochemistry
Brandeis University, MA, USA	Ph.D.	1983	Biochemistry
Whitehead Institute, MA, USA	Postdoctoral	1984-1986	Oncogene

PROFESSIONAL EXPERIENCE: Please list, in chronological order, concluding with present position, previous employment, experience and honors. List, in chronological order, the titles, authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. **DO NOT EXCEED TWO PAGES.**

PROFESSIONAL EXPERIENCE:

1977-1978 Assistant Research Fellow, Institute of Biological Chemistry, Academia Sinica, ROC
1978-1983 Predoctoral Fellow, Department of Biochemistry, Brandeis University, Gillette Graduate Teaching Fellowship
1984-1986 Postdoctoral Fellow, Whitehead Institute/MIT, Postdoctoral fellowship from Cancer Research Institute
1986-1991 Assistant Professor, Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center
1991-1994 Associate Professor, Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center
1994- Professor, Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center
1996- Director, Breast Cancer Basic Research Program, U.T. M.D. Anderson Cancer Center
1996-1999 Hubert L. and Olive Stringer Endowed Professorship in Cancer research
1999-2000 Hubert L. and Olive Stringer Distinguished Professorship in Cancer Research
1999-2000 Chair (Ad interim), Department of Molecular and Cellular Oncology, U.T. M.D. Anderson Cancer Center
2000-2002 Ruth Legett Jones Chair, Department of Molecular and Cellular Oncology, U.T. M.D. Anderson Cancer Ctr.
2000- Chair, Deparment of Molecular and Cellular Oncology, U.T. M.D. Anderson Cancer Center
2003- Ruth Legett Jones Distinguished Chair, Department of Molecular and Cellular Oncology

HONORS & AWARDS:

1989-1990 John P. McGovern Outstanding Teacher Award, U.T. H.S.C.-Houston (1992-1993, 1998-1999)
1993 Faculty Achievement Award in Education (1993) and in Basic research (1998), UTMDACC
1996 Member, PathologyB Study Section (1996-2000) and Sub-committee C (2001-present), NIH
1999 Member, Editorial Board, Clinical Cancer Research (1999-), Cancer Cell (2001-), and Cancer Research (2002-)
2002 Member of the Academia Sinica, Taiwan, ROC

PUBLICATIONS (Peer-reviewed, selected from 212 peer-reviewed publications):

1. Zhou BBP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC (2001): Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nature Cell Biol., 3(3): 245-252
2. Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon LYW, Hung MC (2001): Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nature Cell Biol., 3 (9): 803-808
3. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC (2001): HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nature Cell Biol. 3(11):973-982
4. Hortobagyi GN, Ueno NT, Xia W, Zhang S, Wolf JW, Putnam JB, Weiden, PL, Willey JS, Carey M, Warner DL,

Payne JY, Tucker SD, Barthikimeusz C, Kilbourn RG, De jager RL, Sneige N, Katz RL, Daifuku R, ibarahim NK, Murray JL, Theriault RL, Valero V, Gershenson Dm, Bevers MW, Huang L, Lopez-Berestein G, Hung MC (2001): Cationic liposome-mediated adenovirus type 5 E1A gene transfer to human breast and ovarian cancer cells and its biological effects: A phase I clinical trial. J. Clin. Oncol. 19: 3422-3433
5. Wen Y, Hu MC, Makino K, Spohn B, Bartholomeusz G, Yan DH, Hung MC (2000): HER-2/neu promotes androgen-independent survival and growth of prostate cancer Akt pathway. Cancer Res. 60: 6841-6845
6. Wang SC, Makino K, Su LK, pao A, Kim JS, Hung MC (2001): Ultraviolet irradiation induces depletion of BRCA2 protein through a p53-independent proteosome-independent pathway. Cancer Res. 61: 2838-2842
7. Zou Y, Peng H, Zhou BP, Wen Y, Tsai EM, Wang SC, Hung MC (2002): Systemic tumor suppression by the pro-apoptotic gene bik. Cancer Res. 62: 8-12
8. Deng J, Miller SA, Wang HY, Xia W, Wen Y, Zhou BP, Lin SY, Li YM, Hung MC (2002): b-Catenin interacts with and inhibits NF-kB in human colon and breast cancers. Cancer Cell, 2: 323-334
9. Liao Y, Hung MC (2003): Regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenoviral protein E1A-mediated sensitization to apoptosis. Mol. Cell. Biol., 23: 6836-6848
10. Li YM, Wen Y, Zhou BP, Kuo HP, Ding Q, Hung MC (2003): Enhancement of Bik anti-cancer effect by Bik mutants. Cancer Res. 63: 7630-7633
11. Xia W, Chen JS, Zhou X, Sun PR, Liao Y, Zhou BP, Hung MC (2004): Phosphorylation/cytoplasmic localization of p21 cip1/WAF1 is associated with HER-2/neu overexpression and provides a novel combination predicator for worse prognosis in breast cancer patients. Clin. Cancer Res. 10: 3815-3824
12. Hu MC, Lee DF, Xia W, Golfman L, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC (2004): IkB kinase promotes tumorigenesis through inhibition of Forkhead transcription factor Foxo3a. Cell, 117:225-237
13. Liao Y, Zou Y, Xia W, Hung MC (2004): Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by a non-rival mediated systemic delivery of E1A gene in orthotopic xenograft human breast cancer. Cancer Gene Therapy, 11 (7): 1-9.
14. Wang SC, Lien HC, Xia W, Chen IF, Lo HW, Wang Z, Ali-Seyed M, Bartholomeusz G, Ou-Yang F, Giri DK, Hung MC (2004): Binding at the transactivation of COX-2 promoter by nuclear tyrosine kinase receptor ErbB2. Cancer Cell : in press.
15: Liao Y, Hung MC (2004): A new role of protein phosphatase 2A in adenoviral E1A protein mediated sensitization to anti-cancer drug-induced apoptosis in human breast cancer cells. Cancer Res., September 1, 2004; 64: in press.

Liao, Y

P.I. Last Name, First Initial

BCTR0504146

Tracking ID Number

DISCLOSURE OF HUMAN SUBJECTS/ANIMAL ASSURANCE STATEMENT FORM
(Required in Master Application Packet Only)

Does the proposed work involve human subjects?

☑ No

☐ Yes If YES, verification from an Institutional Review Board MUST be included in the application or on file by March 1, 2005.

Does the proposed work involve human anatomical substances?

☐ No

☑ Yes If YES, please list which anatomical substances will be used. (IRB approval is not required.)

Cell lines derived from human breast cancer tissues.

Does the proposed work involve the use of animals, either by applicant or subcontractor?

☐ No

☑ Yes If YES, verification of the Institutional Animal Care and Use Committee (IACUC) MUST be included in the application or on file by March 1, 2005. Please list which animal subjects will be used.

Balb/c and Balb/c nu/nu mice

Is the final IRB/IACUC approval letter included in the hard copy application?

☑ No

☐ Yes

Principal Investigator Assurance Statement for Animal Care

I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic, and tranquilizing drugs will be used where indicated and appropriate to minimize discomfort and pain to animals.

I assure that the animals authorized for use in this protocol will be used only in the activities, manner, and quantities described herein, unless a deviation is specifically approved by the IACUC for this project and that I will notify the Komen Foundation of this deviation.

I accept full responsibility for the proper care and use of the animals during the conduct of research outlined in the proposal.

I verify that I have made a reasonably good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

I verify that the personnel performing the animal procedures/manipulations described in this protocol are technically competent in those procedures and have received training on the use of animals in research as required by the Animal Welfare Act of 1985.

I assure that I have consulted with an individual qualified to evaluate the statistical design of this proposal and that the minimum number of animals needed for scientific validity are used.

P.I. Signature: Yong Liao

Signature of Institutional Official Typed Name and Title:

Date:

Signature is REQUIRED, even if answered "NO."

PLEASE NOTE:

Final approvals, in hard copy form, must be received by March 1, 2005. Original approvals must be in English, have original signatures, and be mailed to the Foundation.

Liao, Y | BCTR0504146

P.I. Last Name, First Initial | **Tracking ID Number**

CHECK LIST

Basic, Clinical and Translational Breast Cancer Research

Included in Master Packet only. P.I. and an Institutional Official must sign (original signature) hard copy.

BEFORE UPLOADING AND MAILING, ALL APPLICANTS SHOULD CHECK THE SUBMISSION FOR THE FOLLOWING:

TYPE OF APPLICATION:

☑ NEW Application. This application is being submitted to the Komen Foundation for the first time.

☐ REVISED. This application replaces a prior unfunded version of a new application. This is a revision of application number:

Electronic Submission -- The following were uploaded electronically to the Komen Web site:

MASTER APPLICATION PACKET

- ☑ Master Application Cover Page
- ☑ Lay & Scientific Abstract (Uploaded by August 15, 2003)
- ☑ Project Description (New applications: limited to 10 pages including references and figures; Revised applications limited to 12 pages including references and figures.)
- ☑ Budget
- ☑ Budget Justification (limit two pages)
- ☑ Other Sources of Financial Support
- ☑ Biographical Sketch (limit two pages per person)
- ☑ Disclosure of Human Subjects/Animal Assurance Statement Form
- ☑ Check List

REVIEWER APPLICATION PACKET

- ☑ Reviewer Packet Cover Page;
- ☑ Project Description (New applications: limited to 10 pages including references and figures; Revised applications limited to 12 pages including references and figures.) **REMOVE names (whether or not they are part of the research) from the project description and the references page. REMOVE** the name of the P.I., other personnel, collaborators, institutions, collaborating institutions, geographical location, or any other identifying information.
- ☑ Budget. **REMOVE names (whether or not they are part of the research). REMOVE** the name of the P.I., other personnel, collaborators, institutions, collaborating institutions, geographical location, or any other identifying information.
- ☑ Budget Justification (limit two pages). **REMOVE names (whether or not they are part of the research). REMOVE** the name of the P.I., other personnel, collaborators, institutions, collaborating institutions, geographical location, or any other identifying information.
- ☑ Biographical Sketch (limit two pages per person)
- ☑ Disclosure of Human Subjects/Animal Assurance Statement Form - with required P.I. and approving institutional official signature;
- ☑ Check List – with required original signatures
- ☑ Reviewer Packet Cover Page
- ☑ Reviewer Packet Project Description
- ☑ Reviewer Packet Budget Form
- ☑ Reviewer Packet Budget Justification

Hard Copy Submission – The following must be submitted to the Komen Foundation by August 23, 2004, 5 p.m., CDT (one week after the electronic submission);

- ☑ Master Application Cover Page – with required approving institutional official original signature;
- ☑ Lay & Scientific Abstract
- ☑ Project Description
- ☑ Budget
- ☑ Budget Justification
- ☑ Other Sources of Financial Support

Liao, Y

P.I. Last Name, First Initial

BCTR0504146

Tracking ID Number

CHECK LIST SIGNATURE PAGE:

Submission requirements:

I have read the guidelines for the Basic, Clinical and Translational Research Program. I understand if any part of the application I submit is not complete or does not follow the Request for Application Guidelines, the application will not be sent for review, nor will I have the opportunity to correct any out of compliance issues.

Permission to publish:

Permission is hereby given to the Susan G. Komen Breast Cancer Foundation to publish the abstracts submitted in this application if it is selected for funding.

	8-13-04
Principal Investigator's Signature	**Date**
Liao Yong	
Printed Name	
Approving Institutional Official Signature	**Date**
Printed Name/Title	

GENERAL INFORMATION

- **Application must be submitted in English;**
- Electronic abstract must be uploaded to the Komen website at www.komen.org by August 2, 2004, 5 p.m., CDT. Complete electronic applications must be uploaded to the Komen website by August 16, 2004, 5 p.m., CDT. Hard copies must be submitted by August 23, 2004, 5 p.m., CDT (one week after the electronic submission);
- Applications received by fax or email will **NOT** be accepted;
- **DO NOT** send any additional materials;
- If you would like immediate notification of receipt of your application, **send a self address stamped post card;**
- **DO NOT CALL** the Komen Foundation regarding the status of the application during the review period.

Address for hard copy submission and inquiries:

Dianne R. McDonald, Research Grants Manager
The Susan G. Komen Breast Cancer Foundation
5005 LBJ Freeway, Suite 250, Dallas, TX 75244
Phone, fax and email inquiries:
888.300.5582 (Grants Toll Free Line)
972.855.1640 (Fax)
grants@komen.org (Email)

ELECTRONIC ABSTRACTS DUE AUGUST 2, 2004

ELECTRONIC APPLICATION DEADLINE IS AUGUST 16, 2004

HARD COPIES DUE AUGUST 23, 2004