Mitogen-Activated Protein (MAP) Kinases



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Research Article

Raf-1 Activation Prevents Caspase 9 Processing Downstream of Apoptosome Formation

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In many cell types, growth factor removal induces the release of cytochrome-c from mitochondria that leads to activation of caspase-9 in the apoptosome complex. Here, we show that sustained stimulation of the Raf-1/MAPK1,3 pathway prevents caspase-9 activation induced by serum depletion in CCL39/ Δ Raf-1:ER fibroblasts. The protective effect mediated by Raf-1 is sensitive to MEK inhibition that is sufficient to induce caspase-9 cleavage in exponentially growing cells. Raf-1 activation does not inhibit the release of cytochrome-c from mitochondria while preventing caspase-9 activation. Gel filtration chromatography analysis of apoptosome formation *in cells* shows that Raf-1/MAPK1,3 activation does not interfere with APAF-1 oligomerization and recruitment of caspase 9. Raf-1-mediated caspase-9 inhibition is sensitive to emetine, indicating that the protective mechanism requires protein synthesis. However, the Raf/MAPK1,3 pathway does not regulate XIAP. Taken together, these results indicate that the Raf-1/MAPK1,3 pathway controls an apoptosis regulator that prevents caspase-9 activation in the apoptosome complex.

1. Introduction

Apoptosis or programmed cell death is required to maintain cell homeostasis in multicellular organisms [1], its role is essential during embryogenesis as well as in many physiological processes, like immune responses. The components of the apoptotic machinery are expressed in every living cell but remain inactive thanks to environmentally regulated survival signaling pathways [2]. Deregulation of apoptosis control is associated with pathologic processes like neurodegenerative diseases or cancer [3, 4]. Apoptosis execution requires activation of the caspase cascade, which is initiated by two major death-signaling pathways [5]. The extrinsic pathway leads to the activation of caspase 8 after stimulation of cell surface death receptors and the intrinsic pathway, which depends on mitochondrial membrane disruption, leads to caspase 9 activation. Mitochondrial membrane integrity is controlled by the ratio of pro- and anti-apoptotic members of the Bcl2 protein family [6]. The proapoptotic Bcl2 members like Bax Bad or Bim are involved in the release

of apoptogenic proteins such as cytochrome c, smac, and Omi/HTrA2 in the cytosol [7]. Cytosolic cytochrome c binds to apoptotic protease activating factor 1 (APAF-1) and induces the formation of an APAF-1-cytochrome c heptamer complex [8] or apoptosome. This event is regulated by the oncoprotein prothymosin α [9] and heat-shock proteins [10]. Apoptosome recruits and facilitates the autoproteolytic activation of caspase 9 by homodimerization [11]. Once activated, caspase 9 cleaves and activates effector caspases such as caspase 3 and 7 which in turn cleave numerous cellular substrates, finalizing the process of cell death. Caspase 9 activation is controlled by a putative tumor suppressor PHAP [9] and by the binding of X-linked inhibitor of apoptosis (XIAP) [12]. In turn, XIAP is inhibited by the mitochondrial proapoptotic protein Smac/DIABLO and HtrA2/OMI, when they are released into the cytosol [13].

In tissue cell culture, continuous activation of growth factor-stimulated survival pathways is required to prevent apoptosis since growth factors withdrawal induces a metabolic arrest [14] that activates the intrinsic pathway of apoptosis and caspase 9. Among the signaling pathways controlled by serum growth factors, the Raf-1/MAPK pathway has been shown to play important roles in cell survival [15]. Knockout studies in mice reveal that *raf*-1 and b-*raf* are involved in developmental cell survival [16]. In human, constitutive activation of MAPK1,3 pathway by Ras or B-Raf oncogenes, is implicated in inhibition of apoptosis in tumor cells, especially in leukemia and melanoma [17, 18].

The MAPK1,3 pathway is involved in the control of Bcl2 family proteins. MAPK1,3 activation has been found to increase the expression of the anti-apoptotic proteins Bcl2, BclXL, and Mcl-1 [19, 20] by transcriptional and posttranslational mechanisms. On the other hand, the MAPK1,3 pathway inactivates proapoptotic members like Bad and Bax by phosphorylation [21] and enhances degradation of Bim by the proteasome [22]. The Raf-1/MAPK1,3 pathway has also been implicated in caspase inhibition downstream of cytochrome c release [23-25]. A potential target of MAPK1,3 is caspase 9 whose activation in vitro is inhibited through direct phosphorylation at Thr 125 by MAPK1 [26]. Caspase 9 was shown to be transiently phosphorylated upon MAPK activation in intact cells but there is not yet evidence that this phosphorylation event prevents the processing of the proform during apoptosis induction. In CCL39 △Raf-1:ER cells, we and others have shown that sustained activation of MAPK1,3 pathway inhibits matrix detachment and serum deprivation-induced apoptosis [27, 28].

In this study, we further investigated the mechanism by which MAPK1,3 activation blocks serum deprivationinduced caspase 9 cleavage. We show that caspase 9 inhibition requires MEK activity and protein synthesis. This antiapoptotic effect occurs downstream of cytochrome c release and does not interfere with APAF-1 oligomerization or caspase 9 recruitment into high molecular weight complexes. Furthermore we show that the expression levels of XIAP do not correlate with protein synthesis-dependent caspase 9 inhibition. These results indicate that the Raf-1/MAPK1,3 pathway blocks caspase 9 activation in the apoptosome.

2. Material and Methods

2.1. Reagents. The MEK inhibitor U0126 was obtained from Promega (Madison, WI), PD98059, emetine, 4hydroxytamoxifen (4-HT) and the mouse monoclonal antiactivated MAPK (Sigma M8159; 1:10,000) were from Sigma (St. Louis, MO). PD184352 was kindly provided by Professor P Cohen (MRC University of Dundee, UK). The monoclonal anti-poly (ADP-ribose) polymerase (anti-PARP) anti-body (Signal Transduction SA-250 1:2,000) and caspase 9 peptide substrate Ac-LEHD-pNA were purchased from Biomol (Plymouth Meeting, PA). Other anti-bodies used include rabbit polyclonal anti-MAPK1 (1:5,000) [29], mouse monoclonal anti-denaturated cytochrome c (Pharmingen BD biosciences San Jose CA, 556433, 1:2,000), rabbit polyclonal anti-active caspase 3 (Cell Signaling Technology Danvers MA, 9661 1:200), mouse monoclonal anti-caspase 9 (MBL Woburn MA M054-3 1:2,000) and mouse monoclonal anti-XIAP (Transduction Laboratories BD biosciences San Jose CA, 1:250).

2.2. Cell Culture. CCL39- Δ Raf-1:ER cells (clone S18) [30] Chinese hamster fibroblasts stably express an estradiolregulated form of oncogenic Raf-1 kinase (plasmids were kindly provided by Dr. M. McMahon, University of California San Francisco Cancer Center, San Francisco, CA). CCL39- Δ Raf-1:ER cells were cultivated in DMEM (Life Technologies, Gaithersburg, MD) without phenol red and supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml), 7.5% fetal calf serum (FCS), and G418 (400 µg/ml).

2.3. SDS-PAGE and Western Blot Analysis. Adherent and floating cells were lysed in Laemmli sample buffer, sonicated 15 seconds, and incubated at 65°C for 15 minutes. This protocol, recommended by the manufacturer of the anti-PARP anti-body for detection of PARP cleavage, was used for all immunoblot experiments. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore Billerica MA). Dry blots were blocked in 3% dry milk (dissolved in water for 10 minutes at room temperature), incubated overnight at 4°C with the primary anti-body, washed 5 minutes with tap water. After blocking again, membranes were incubated 1 hour at 4°C with horseradish peroxidase—(HRP, 1:40,000) or alkaline phosphatase—(AP, 1:2,000) conjugated secondary anti-bodies (Cell Signaling Technology, Beverly, MA). Immune complexes were detected by autoradiography following enhanced chemiluminescence with SuperSignal (Pierce Thermo Fisher Rockford IL) for HRP or CDP-star (NEB Beverly, MA) for AP.

2.4. Fractionation by Gel Filtration. The cell lysates (100,000 g supernatant) were prepared by resuspending cells in a buffer containing 50 mM PIPES/KOH, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol and a protease inhibitor cocktail (Roche). After a freeze/thaw cycle in liquid nitrogen, the lysates were fractionated by size-exclusion chromatography using a fast protein liquid chromatography protein purification system (Amersham Pharmacia Biotech GE Buckinghamshire UK) on an analytical (16/60) Hi Prep S300 Sephacryl high-resolution column (Amersham Pharmacia Biotech). The column was pre-equilibrated with 5% (w/v) sucrose, 0.1% (w/v) CHAPS, 20 mM HEPES, 5 mM dithiothreitol, pH 7.0. All separations were carried out at room temperature. The column was calibrated with gel filtration protein standards from Sigma (thyroglobulin MW = 669,000, ferritin MW = 443,000, β amylase MW = 200,000, alcohol dehydrogenase MW = 150,000, bovine serum albumin MW = 66,000).

Lysates (5 mg of protein) were applied to and eluted from the column at a flow rate of 0.4 ml/min, 500 μ l fractions were collected and stored at -70° C.

2.5. Enzymatic Assay for Caspase 9 Activity. Cell lysates (100,00 g fraction of supernatant) were prepared by resuspending cells for 20 minutes at 4°C in buffer containing 0.1% (w/v) CHAPS, 0.5% Nonidet P-40, 50 mM HEPES pH 7.4, 100 M NaCl, 1 mM EGTA, and 10 mM dithiothreitol. Cell lysates (200 μ g protein) were incubated with 200 μ M caspase 9 peptide substrate Ac-LEHD-*p*NA at 37°C for 1 hour.

The *p*NA light emission was quantified using a microtiter plate reader at 405 nm (Labsystems iEMS Reader MF Helsinky Finland).

2.6. In Vitro Activation of Caspase 9. Cell lysates were prepared by resuspending cells for 20 minutes at 4°C in buffer A (20 mM HEPES pH 7.4 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, and 5 mM dithiothreitol) followed by centrifugation at 10,000 g for 20 minutes. Aliquots of the supernatant containing 5 mg/ml protein were frozen in liquid nitrogen and kept at -70° C. Activation of caspase 9 was induced by the addition of 1 μ M bovine heart cytochrome c (Sigma St Louis MI) and 1 mM ATP (Fermentas Thermo Fisher Rockford IL) to 20 μ l cell lysate and incubated at 20°C, the reaction was stopped by addition of 2% SDS.

2.7. Immunofluorescence Assays. Cells were fixed in 3% paraformaldehyde, permeabilized with PBS/0.2% Triton and blocked with PBS/10% FCS. Cells were stained for 1 hour with an anti-cytochrome c monoclonal mouse anti-body (Pharmingen 556432 1:200) and with an anti-active caspase 3 rabbit polyclonal anti-body (Cell Signaling 9661 1:200). Cells were washed twice with PBS and blocked again with PBS/10% FCS before staining for 1 hour with Alexa-594 coupled anti-mouse anti-body, Alexa-488 coupled antirabbit anti-body from Molecular Probes (Eugene, OR). Nuclei were stained for 15 minutes with 50 ng/ml 4',6diamidino-2-phenylindole dihydrochloride (DAPI; Roche, Hertforshire, United Kingdom). Fluorescence was observed on a Zeiss inverted microscope (Axiovert 200 M) equipped with a CoolSnap HQ cooled charge-coupled-device camera (Roper Scientifique, Every, France). Image acquisition and analysis was performed using the MetaMorph Imaging System (Universal Imaging Corp. Buckinghamshire UK).

2.8. Caspase 9 Expression Vectors. Caspase 9 was cloned in Topo vector (Invitrogen) following RT-PCR amplification from HeLa cell total RNA. PCR primers flanking the coding sequence were designed from the sequence U60521 (genbank) [31] and correspond to nt14–34 AGGCGGCCTG-GAGTCTTAGTT and nt1311-1329 ACCCTGCCTTAT-CTTGCAC. A BamHI restriction site was introduced by PCR at the initiation codon and the BamHI/EcoRI insert was cloned in pCMV-tag 3B (Stratagene) to introduce the tag myc in N-terminal. A potential MAPK1,3 phosphorylation site at Thr 125 was detected using the Phosphobase algorithm at high stringency http://phospho.elm.eu.org/. The threonine was replaced by an alanine using Quick Change mutagenesis kit (Quiagen Hilden Germany). To derive stable cell lines expressing these constructs, the catalytic cysteine (C287) was replaced by a serine. Transfections were performed by the DNA-calcium phosphate coprecipitation method.

2.9. 2D Gel Analysis. Cells were lysed with 1 vol 10 mM Tris, 1 mM EDTA, 0.5% CHAPS containing phosphatase and protease inhibitors. Proteins were precipitated by addition of 3 vol acetone at -20° C and collected at 10000 g for 10

minutes. The pellets were resuspended at 10–20 mg/ml in IEF sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 40 mM Tris and ampholytes). The first dimension was performed in a Zoom IPG Runner (Invitrogen) on pH 4–7, 7 cm strips. The transfer blots and corresponding autoradiograms were aligned using amido black staining of the PI calibration markers (creatine phosphokinase carbamylyte, Amersham pharmacia Biotech Buckinghamshire UK).

3. Results

3.1. Raf-1 Activation Prevents Caspase 9 Cleavage upon Serum Withdrawal. In exponentially growing CCL39-ARaf-1:ER cells, sudden growth factor deprivation triggers apoptosis. Although interactions with components of the extracellular matrix provide anti-apoptotic signals to these adherent fibroblasts, serum removal for 24 hours results in more than 50% cell death, as determined by propidium iodide staining (not shown). CCL39-△Raf-1:ER cells express the fusion protein \triangle Raf-1:ER, therefore, addition of 4-hydroxytamoxifen (4-HT) results in the selective and persistent activation of Raf-1 signaling. 4-HT addition to serum-deprived cells leads to a complete inhibition of apoptosis (data not shown, [27, 28]). As shown in Figure 1(a), serum removal gradually decreased the levels of phosphorylated MAPK1 and 3 (also known as ERK1 and 2) and this was accompanied by the appearance of the p35 and p37 cleavage products of procaspase 9. The presence of 4-HT maintained phosphorylation of MAPK1,3 and completely inhibited caspase 9 cleavage.

Consistent with the fact that procaspase 9 cleavage is generally associated with the generation of the active form of caspase 9, we observed an increase in caspase 9 activity in cell extracts prepared 14 hours after serum removal and this activity was significantly inhibited in the presence of 4-HT (Figure 1(b)). Caspase 3 is known to be a direct substrate of caspase 9, accordingly its activation was also found to be repressed upon Raf-1 stimulation (Figure 1(b)). These results indicate that sustained Raf-1 activation inhibits caspase 9 cleavage and activity following serum removal thereby preventing apoptosis to proceed.

3.2. Caspase 9 Activation Is Inhibited by the MEK/MAPK1,3 Pathway. To determine the role of Map kinases in the inhibition of caspase 9 cleavage, 4-HT-treated cells were incubated with increasing concentrations of the specific MEK1 inhibitors U0126, PD98059 and PD184352. All of these pharmacological agents prevented Raf-1-induced MAPK activation and reversed the cleavage of caspase 9 (Figure 2(a)). 4-HT-induced Raf-1:ER activation is dose-dependent, the dose-response experiment shown in Figure 2(b) clearly indicates a close relationship between MAPK phosphorylation and caspase 9 cleavage. It should be noted that in exponentially growing cells, the inhibition of MEK by U0126 was sufficient to induce a weak cleavage of caspase 9 (Figure 2(c)), which is more pronounced at lower serum concentration. Thus, we can conclude that the inhibition of caspase 9 cleavage by Raf-1 depends on MEK activity



FIGURE 1: (a) Raf-1 activation inhibits caspase-9 cleavage upon serum withdrawal. CCL39- Δ Raf-1:ER cells treated with or without 4-HT were deprived of serum for the indicated time periods. The cell lysates were analyzed by immunoblotting for caspase-9, phospho-MAPK1,3, and total MAPK1. (b) Raf-1 activation inhibits caspase activity CCL39- Δ Raf-1:ER-cells treated or not with 4-HT were deprived of serum for 14 hours as indicated. sts: the effect of 0.3 mM staurosporin is shown as a comparison. Caspase 9 activity was measured using the peptide LEHD-pNA as a substrate and caspase 3 activity using DEVD-pNA. The mean values \pm SEM were calculated from at least two separate experiments performed in triplicate.

and MEK activity contributes permanently to the protection against caspase 9 activation provided by serum.

3.3. Caspase 9 Inhibition Requires Protein Synthesis and Persistent MEK Activity. We then determined if the protective effect of Raf-1 would require de novo protein synthesis. Because cycloheximide has been shown to completely prevent serum deprivation-induced apoptosis in CCL39derived lines (our unpublished observations and [28]), we analyzed the effect of emetine, another protein synthesis inhibitor. As shown in Figure 3(a), addition of emetine at the same time as 4-HT (lane 3, emetine at time 0) completely blocked Raf-1-mediated caspase 9 inhibition, without affecting Raf-1-induced phosphorylation of MAPK. Raf-1-mediated protection was still ineffective when emetine was added two hours after 4-HT stimulation and was gradually restored when emetine was added later. Addition of emetine 8 hours after Raf-1 activation (last lane Figure 3(a)) did not affect inhibition of caspase 9 cleavage anymore since only full-length procaspase 9 is detected. These results indicate that Raf-1-mediated caspase 9 inhibition requires protein synthesis and that complete protection is achieved after 6-8 hours accumulation of a caspase 9 inhibitor. XIAP is one of the potential inhibitors of caspase 9 downstream cytochrome c release. Thus, we investigated the expression of XIAP following 4-HT treatment of CCL39-∆Raf-1:ER cells and found that XIAP expression was not affected by Raf-1 activity or by emetine treatment (Figure 3(a)). These results indicate XIAP is not the emetine-sensitive inhibitor of caspase 9.

Caspase 9 processing have been shown to be regulated *in vitro* by direct MAPK1,3 phosphorylation [26], to determine if Raf-1/MAPK-mediated protection of caspase 9 cleavage

in intact cells was only a posttranslational mechanism we determined the time course of Raf-1 action. Figure 3(b) shows that delayed addition of U0126 after 4-HT as a mean to terminate Raf-1/MAPK action resulted in caspase 9 cleavage. When U0126 was added 12, 10, or 8 hours after 4-HT and the experiment terminated at 24 hours, the cells remained unprotected for 12, 14, and 16 hours, respectively. Under these conditions, the extent of caspase 9 cleavage was similar to that in cells that were maintained unprotected for the same times because of delayed addition of 4-HT (Figure 3(c)) This result indicates that 4HT preincubation is not sufficient to prevent caspase 9 cleavage. Thus, caspase 9 protection requires both protein synthesis and continuous Raf-1 signaling.

3.4. Raf-1/MAPK Activation Induces Posttranslational Modifications of Caspase 9. The above-mentioned experiments suggest the involvement of posttranslational mechanisms in the protection of caspase 9 cleavage, we thus investigated the role of the recently identified MAPK1,3 phosphorylation site T125 present on caspase 9. Figure 4(a) shows the transient expression of a myc tagged mutated form of caspase 9 in which T125 is substituted by the nonphosphorylatable residue alanine. Serum withdrawal-induced cleavage of caspase 9 T125A was totally prevented by 4-HT addition. This results indicates that phosphorylation of T125 is not involved in Raf-1/MAPK protection of caspase 9 cleavage.

In addition, two-dimensional electrophoresis analysis of catalytically inactives capase 9 and mutant caspase 9 after Raf-1 stimulation (Figure 4(b)) revealed that caspase T125A was still able to shift in response to 4-HT addition. This result suggests the presence of an additional phosphorylation site on caspase 9. In the presence of 4-HT, caspase 9 clearly



FIGURE 2: Raf-1-mediated caspase-9 inhibition depends on MEK activity. (a) CCL39- Δ Raf-1:ER cells were serum deprived for 24 hours in the absence or presence of 1 μ M 4-HT. The indicated concentrations of the MEK inhibitors PD98059 and PD184352 were added 30 minutes before 4-HT addition. The cell lysates were analyzed by immunoblotting for caspase-9, phospho-MAPK1,3, and total MAPK1. (b) Dose response for U0126 at different 4-HT concentrations. (c) Exponentially growing CCL39- Δ Raf-1:ER cells in the presence of 5% or 1% fetal calf serum were treated for 24 hours with increasing concentrations of U0126.

showed an additional spot indicating that indeed T125 is phosphorylated in response to 4-HT. Both sites were sensitive to U0126.

3.5. Raf-1 Activation Does Not Prevent Cytochrome c Release. The release of cytochrome c from mitochondria into the cytosol is an important cellular event leading to caspase 9 processing. We, therefore, examined the distribution of cytochrome c following serum removal in control and 4-HT-treated cells by immunofluorescence analysis. As shown in Figure 5(a), control cells presented a typical punctuate staining of cytochrome c corresponding to its mitochondrial localization. After 14 hours of serum removal, 20% of the cells exhibited a rounded morphology (cell marked with asterisk on Figure 5(a)). Only the cells that remained adherent were examined, and since apoptosis induces cell detachment, these values are only indicative of the cell population status at a given time point. Rounded cells presented a diffuse cytochrome c staining and an intensification of active caspase 3 labeling. Since caspase 3 is a direct caspase 9 substrate, the presence of active caspase 3 is indicative of caspase 9 activation. Nuclear condensation was detected in 50% of these cells, indicating that half the cells which released cytochrome c, completed apoptosis 14 hours after serum withdrawal. Interestingly, although 4-HT treatment inhibited caspase 3

activation and nuclear condensation after serum removal, it did not interfere with cytochrome c release (cell marked with arrow on Figure 5(a)). Rather, an increase in cells exhibiting cytoplasmic cytochrome c was detected in 4-HT-treated cells compared to nontreated cells (Figure 5(b)); this finding indicates that Raf-1/MAPK prevents apoptosis to proceed in the presence of cytosolic cytochrome c. The activation of Raf-1 did not perturb mitochondrial localization in control, serum-stimulated cells (not shown).

To confirm that caspase 9 activation can be inhibited in the presence of cytosolic cytochrome c, we performed in vitro assays. Cytosolic extracts from cells deprived of serum for 9 hours in the presence or absence of 4-HT were incubated with 1 µM cytochrome c and 1 mM ATP [32]. Incubation was performed at 20°C in order to avoid the rapid inactivation of MAPK observed at 37°C (not shown). As can be seen in Figure 6, cytochrome c/ATP addition induced caspase 9 cleavage in serum-deprived cell extracts but not in extracts from 4-HT treated cells. A similar result was obtain upon electroporation of purified cytochrome c in intact cells that were preincubated with 4-HT (see Figure S1 in Supplementary material available online at doi:10.1155/2011/834948). These results further demonstrate that Raf-1 activation inhibits caspase 9 cleavage downstream of mitochondrial cytochrome c release.



FIGURE 3: (a) Raf-1-mediated protection of caspase 9 cleavage requires protein synthesis. CCL39- Δ Raf-1:ER cells were deprived of serum for a total time of 24 hours in the presence or absence of 1 μ M 4-HT. The protein synthesis inhibitor emetine (20 μ M) was added at the indicated time point. The cell lysates were analyzed by immunoblotting for caspase-9, phospho-MAPK1,3, total MAPK1 and XIAP. (b) and (c) time course of Raf-1-mediated caspase 9 inhibition. (b) CCL39- Δ Raf-1:ER cells were deprived of serum for a total time of 24 hours in the presence of 50 nM 4-HT. 10 μ M U0126 was added at the indicated time after serum withdrawal. (c) Cells were deprived of serum for 24 h; 4-HT (1 μ M) was added at the indicated time after serum withdrawal. The cell lysates were analyzed by immunoblotting for caspase-9.

3.6. Raf-1 Activation Does Not Prevent Apoptosome Formation. In the cytosol of apoptotic cells, binding of cytochrome c to APAF-1 leads to its oligomerization and recruitment of procaspase 9 into a high molecular weight complex known as the apoptosome [8]. We examined whether Raf-1 activation inhibited procaspase 9 activation in the cells by blocking formation of the apoptosome. To do so size exclusion chromatography was performed on cytosolic extracts of CCL39- Δ Raf-1:ER cells followed by Western blot analysis of



FIGURE 4: Mutation of threonine 125 does not prevent caspase 9 phosphorylation and cleavage inhibition. (a) CCL39- Δ Raf-1:ER cells were transfected with the indicated construct (CD8 is used as a transfection control) and 48 hours later were deprived of serum in the presence or absence of 1 μ M 4-HT for 24 hours. The cell lysates were analyzed by immunoblotting for myc and phospho-MAPK1,3. (b) CCL39- Δ Raf-1:ER cells stably expressing myc-tagged C287S (catalytically inactive) caspase 9 or C287S-T125A caspase 9 were incubated for 2 hours with 1 μ M 4-HT or 10 μ M U0126 in serum-free medium. Total cells were lysed in 2D sample buffer containing 6 M urea, resolved by two-dimensional gel electrophoresis, and analyzed by immunoblotting for myc.

the different apoptosome components. Results in Figure 7(a)show that in lysates from control cells in presence of serum, APAF-1 was located in fractions corresponding to M_r of ~200 kDa–400 kDa. In lysates from serum-deprived cells, the APAF-1 signal shifted to fractions corresponding to approximately M_r of ~700 kDa, consistent with the formation of an active apoptosome complex in vivo [33]. In extracts from serum-stimulated cells, cytochrome c was not detected in fractions containing high molecular weight complex. However, following serum removal, cytochrome c coeluted with APAF-1 in high molecular weight fractions (Figure 7(b)). Raf-1 activation does not interfere with the relocalization of APAF-1 and cytochrome c into high molecular weight complexes upon serum withdrawal (Figures 7(a) and 7(b)), these results suggest that Raf-1 activation does not interfere with the apoptosome formation. The presence of cytochrome c in the high molecular weight fractions also confirm that Raf-1 activation does not block cytochrome c release. We then determined if caspase 9 present in living



FIGURE 5: (a) Raf-1 activity does not prevent cytochrome c release. CCL39- Δ Raf-1:ER cells were deprived of serum for 14 hours in the presence or absence of 4-HT. Fixed and permeabilized cells were stained for cytochrome c and active caspase-3. Nuclei were stained with DAPI. Arrows show cells with cytochrome c released. (b) Quantification of Figure 5(a): Cytochrome c release and apoptotic nuclei were quantified from 200 adherent cells in triplicates.



FIGURE 6: Raf-1 activation prevents cytochrome c/ATP-mediated activation of caspase 9. CCL39- Δ Raf-1:ER cells were deprived of serum for 9 hours in the presence or absence of 4-HT. The cells were lysed in hypotonic buffer and the cell lysates were incubated with 1 μ M cytochrome c and 1 mM ATP for the indicated times. The cell lysates were analyzed by immunoblotting for caspase-9, phospho-, and total MAPK1,3.

cells was recruited into the apoptosome (Figure 7(c)) and found that under all conditions, most of the caspase 9 signal was detected in fractions corresponding to M_r of ~60 kDa to 150 kDa. This in contrast to results obtained after activation in cell extracts where the cleaved caspase 9 is mainly present in the high molecular weight fractions (see Supplementary Figure S2) and indicates that the active caspase 9 is rapidly released from the apoptosome in vivo. However, a small caspase signal could be detected in the high molecular weight fractions that could be enhanced by concentrating the eluat. As shown in Figure 7(d), a higher resolution of high molecular weight fractions revealed the presence of caspase 9. Some procaspase 9 was found in the ~700 kDa fractions of serum containing cell, while its amount increased notably in high molecular weight fractions from serum-deprived cells. The ~700 kDa fractions from serum-starved cells also contained the cleaved form of caspase 9. In 4-HT-treated serum-deprived cells, a large amount of procaspase 9, but not cleaved caspase 9, was found in the ~700 kDa fractions. These results indicate that in intact CCl39 cells, the majority of caspase 9 is not associated with APAF-1 and that Raf-1 activity, while preventing caspase 9 cleavage, does not inhibit the pool of caspase 9 that is transiently recruited into the apoptosome.

Altogether, these results indicate that the Raf-1/MAPK1,3 pathway controls the cleavage of caspase 9 within the apoptosome.

4. Discussion

Growth factor deprivation is often linked to activation of the mitochondrial pathway of apoptosis because overexpression



FIGURE 7: (a) and (b): Raf-1 activity does not interfere with apoptosome formation. CCL39- Δ Raf-1:ER cells growing in 7.5% FCS (control) were deprived of serum (-S) for 14 hours in the presence or absence of 4-HT. The lysates were separated by gel filtration chromatography on a sephacryl S300 column previously calibrated with the indicated molecular weight standards. Samples from high molecular weight fractions were diluted with 4X Laemmli buffer and were analyzed by immunoblotting for the presence of APAF-1 (a) and cytochrome c (b). (c) and (d): recruitment of caspase 9 in the apoptosome. (c) The conditions are the same as in Figures 7(a) and 7(b); samples are a pool of 2 fractions and were analyzed by immunoblotting for caspase 9. pro: procaspase 9, 47 kd. p37, p35: cleaved caspase 9. (d) Recruitment of caspase 9 in high molecular weight fractions. Detailed analysis of high molecular weight fractions from Figure 7(c).

of Bcl2 prevents caspase activation. However, very few reports have documented caspase 9 activation in intact cells. In this paper, we clearly show that caspase 9 is activated and cleaved following serum deprivation of exponentially growing CCL39 cells. Activation of Raf-1:ER by 4-HT leads to the specific activation of the MEK/MAPK1,3 signaling module [27] and prevents caspase 9 activation. A panel of MEK inhibitors inhibits the effects of Raf-1:ER. Since U0126 and PD98059 also inhibit MEK5/ERK5, a pathway involved in Raf-1 signaling [34], we used PD184161 a specific MEK1,2 inhibitor [35] at low concentrations [36] to show that the MEK/MAPK1,3 pathway mediates caspase 9 inhibition. Treatment of exponentially growing CCL39 cells by MEK inhibitor was sufficient to cause the appearance of the p35 and p37 processed caspase 9 products. This finding suggests that MEK activity participates in serum-mediated cell protection and that high levels of MAPK activity are continually required to maintain caspase 9 inactive. Serum deprivation of CCl39 cells has often been used to study cell cycle regulation [37]. To arrest CCL39 cells in G0/G1 and limit cell death upon serum deprivation, cells were usually grown to confluency during several days, the progressive limitation in nutrients resulting in an adaptation of cells to growth factor deprivation. Growth factor deprivation affects nutrient utilization and induces a metabolic arrest that results in apoptosis [14]. It is thus possible that sudden growth factor deprivation has a more pronounced effect in actively growing cells than in quiescent cells with limited nutrient needs. Indeed, most quiescent cells in serumdeprived medium are alive and can reenter the cell cycle, while about 65% of an actively growing cell population dies upon serum removal. This observation might be relevant to tumor cell biology since tumor cells need to maintain a high metabolic rate with limited survival factors. Pharmacological inhibition of MEK activity has been shown to induce cell death in exponentially growing cell lines derived from melanoma [38], leukemia [17], fibrosarcoma, and renal carcinoma [39]. It remains to be determined if MEK inhibitors would induce caspase 9 cleavage in tumor cells.

Our immunofluorescence and in vitro caspase 9 activation assays show that Raf-1-mediated inhibition of caspase 9 occurs at a postcytochrome c release level. The ability of MAPK pathway to prevent apoptosis progression following cytochrome c release has been described in several other cell models. For example, △Raf1:ER activation inhibits cell detachment-induced apoptosis in MDCK cells [40] and MEK inhibits caspase 3 activation in serum-deprived Rat1 fibroblasts [23] or in etoposide-treated small cell lung cancer cells [24]. Moreover, MEK activity also blocks cytochrome cinduced caspase 3 activity in Xenopus and Rat1 fibroblast cell free extracts [23, 41]. In vivo, acidic pH induced a very similar mechanism of inhibition of caspase 9 processing without impaired cytochrome c release or APAF-1 interaction [42]. NGFs have been shown to mediate neuron resistance to apoptosis in response to cytochrome c microinjection in the cytosol [43]. This observation led to the notion of a "competence to die" induced by growth factor withdrawal; our results indicate that Raf-1/MAPK inactivation would

render cells competent to respond to cytosolic cytochrome c and then to die.

There is a large body of evidence indicating that MAPK1,3 signaling is involved in the regulation of mitochondria homeostasis. For instance, p90 rsk phosphorylates and inactivates the proapoptotic protein, Bad [21, 44] while direct phosphorylation of the anti-apoptotic protein Bcl2 by MAPK1,3 results in its stabilization and enhanced cell survival [19]. These findings indicate that the MAPK1,3 pathway inhibits apoptosis by preventing cytochrome c release, an hypothesis that has not been directly documented. Indeed, CCL39 cells are sensitive to the anti-apoptotic properties of Bcl2 family proteins because stable expression of BclXl prevents caspase activation upon anchorage and serum withdrawal (our unpublished observations). Thus, under our stringent conditions of apoptosis induction, the putative control of mitochondrial integrity by MAPK1,3 is not efficient enough to prevent cytochrome c release. In CCL39 △Raf1:ER cells, Cook and colleagues clearly demonstrated that MAPK-induced degradation of Bim by the proteasome resulted in Bax inactivation [28, 45]. However, downregulation of Bim has not been shown to inhibit cytochrome c release in CCL39 cells, an event that could be regulated by other MAPK-insensitive BH3-containing proteins.

Our analysis of apoptosome by gel filtration chromatography indicated that the protective effect of MAPK1,3 pathway does not interfere with APAF-1 multimerization or caspase 9 recruitment. However, even in apoptotic conditions, most of the caspase 9 was located in low molecular weight fractions. Most of the publications illustrating the recruitment of caspase 9 in the apoptosome are performed in vitro [46] in the presence of exogenously added cytochrome c and ATP and it is not known how much caspase 9 is associated with multimerized APAF-1 in vivo. Since present models of caspase 9 activation indicate that only a minor portion of purified caspase 9 is found in the active dimeric form [12] it is perhaps not surprising to find little caspase 9 in high molecular weight complexes in vivo. However, when Raf-1/MAPK is activated there is a clear increase in the recruitment of the procaspase 9 to the 700 kDa complex. Recruitment of procaspase 9 is thought to favor a homodimerization step where a monomer provides, the structural constraints required for the constitution of a functional catalytic cleft [47]. This subsequently results in the autoproteolysis of the link between the large and the small catalytic subunits. Since caspase 9 is not cleaved in 4-HTtreated cells, we can conclude that Raf-1 activation is likely to interfere with the formation of an active dimer.

Our results indicate that protein synthesis is required for caspase 9 inhibition. Among the potential inhibitors of the apoptosome, heat-shock proteins are not likely to be involved in Raf-1-induced caspase 9 inhibition, because they interfere either with APAF-1 oligomerization or caspase 9 recruitment [48, 49]. Similarly, an other negative regulator of apoptosis, the oncoprotein prothymosin α is not a likely mediator of Raf-1 effects because it prevents apoptosome formation [9] however, the same study identified PHAP as positive regulator of caspase 9 activation in the apoptosome. It is not

yet known whether proteins of the PHAP family are required for caspase 9 activation in intact cells and then would be regarded as potential targets of Raf-1/MAPK activity. The major inhibitor of caspase 9 is XIAP. XIAP inhibits the catalytic activity of caspase 9 by using its BIR3 domain to heterodimerize with a caspase 9 monomer through the same interface that is required for the caspase 9 homodimerization [12]. However, caspase 9/XIAP interaction also requires the cleavage of caspase 9 because the newly generated small subunit N-terminal stabilizes the interaction [50]. Since caspase 9 is not cleaved in the presence of activated Raf-1, a stable interaction between caspase 9 and XIAP is not expected, however, overexpression of XIAP by inhibiting newly generated cleaved caspase 9 would prevent further autoprocessing of procaspase monomers In mammals, MAPK activation has been associated with the increase of XIAP expression in melanoma cells [51], monocytes [52] and Jurkat cells [53]. In small-cell lung cancer cells, Downward and colleagues have demonstrated that FGF2 inhibits etoposide-mediated caspase 3 activation downstream cytochrome c [24]. It was found that MAPK1,3 activation by FGF2 inhibits the release of Smac from mitochondria. Since Smac triggers the proteasomal degradation of XIAP, MAP activation resulted in XIAP stabilization. In our hands, XIAP was not induced following Raf-1 activation, as it would be expected if Smac were retained in the mitochondria. Moreover, XIAP levels were insensitive to emetine, an indication that XIAP is not the emetine sensitive anti-apoptotic protein required for Raf-1mediated caspase 9 protection. We cannot exclude that Raf-1 activity up regulates XIAP anti-apoptotic effect by inducing a cofactor that could enhance caspase 9/XIAP interaction; however, we were not able to coimmunoprecipitate caspase 9 and XIAP in cell extracts from 4-HT treated cells (data not shown).

Clarke and colleagues reported that MAPK pathway directly controls caspase 9 activation in vitro by phosphorylation on its residue Thr125. Although Thr125 phosphorylation of caspase 9 through a MEK-dependent mechanism occurs in PMA treated HeLa cells [26], it has not yet been linked with cell survival. In serum-deprived CCL39 cells expressing a human caspase 9 mutated on its phosphorylation site (caspase 9 T125A), we show that MAPK activation is still able to inhibit the cleavage of this mutant. Thus, it may be argued that phosphorylation of caspase 9 on threonine 125 is not involved in MAPK-mediated caspase 9 inhibition in serum-deprived cells. However, we cannot exclude that the inhibition of endogenous caspase 9 by phosphorylation on T125 interferes with the cleavage of the mutated form within heterodimers despite the fact that caspase 9 T125A was overexpressed by transient transfection. Our 2D gel analysis indicates that caspase 9 T125A still shifts in response to Raf-1 activation, an indication that it is phosphorylated on another residue. We can exclude S196 a site phosphorylated by AKT [54] because this site present on human caspase 9 is not conserved in rodents [55] and Raf-1:ER does not activate Akt in CCl39 cells [27]. Caspase 9 is also phosphorylated by PKA [56], but since PKA is not inhibited by U0126 [57], it is not likely to be involved because U0126 totally prevents caspase shifts on 2D gels. However, we cannot exclude that this

MEK-dependent charge shift results from the synthesis or processing of an autocrine ligand [58] that does not activate the PI3K pathway [27], yet activates other sigalling pathway leading to caspase 9 phosphorylation (reviewed in [59]).

The role of caspase 9 phosphorylation remains to be clarified, since procaspase 9 activation requires an obligatory dimerization step provided by homophilic contact between the catalytic subunits, this region of the caspase could be a site of interaction with a putative inhibitor of dimerization or a site of direct phosphorylation.

Abbreviations

FCS: Fetal calf serum

- MAPK: Mitogen-activated protein kinases 1, 3
- 4-HT: Hydroxytamoxifen.

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Review Article

Activation of Mitogen-Activated Protein Kinase in Descending Pain Modulatory System

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The descending pain modulatory system is thought to undergo plastic changes following peripheral tissue injury and exerts bidirectional (facilitatory and inhibitory) influence on spinal nociceptive transmission. The mitogen-activated protein kinases (MAPKs) superfamily consists of four main members: the extracellular signal-regulated protein kinase1/2 (ERK1/2), the c-Jun N-terminal kinases (JNKs), the p38 MAPKs, and the ERK5. MAPKs not only regulate cell proliferation and survival but also play important roles in synaptic plasticity and memory formation. Recently, many studies have demonstrated that noxious stimuli activate MAPKs in several brain regions that are components of descending pain modulatory system. They are involved in pain perception and pain-related emotional responses. In addition, psychophysical stress also activates MAPKs in these brain structures. Greater appreciation of the convergence of mechanisms between noxious stimuli- and psychological stress-induced neuroplasticity is likely to lead to the identification of novel targets for a variety of pain syndromes.

1. Introduction

In human brain, there is a neural network that modulates the transmission of nociceptive messages, which is termed descending pain modulatory system. The cerebral cortex and amygdala project directly and indirectly via the hypothalamus to the periaqueductal grey (PAG). The PAG in turn controls spinal nociceptive neurons through relays in the rostral ventromedial medulla (RVM) and the dorsolateral pontine tegmentum (DLPT). The RVM consists of the nucleus raphe magnus (NRM), nucleus reticularis gigantocellularis pars alpha (GiA), and the ventral nucleus reticularis gigantocellularis (Gi) and is a major source of serotonergic projections to the spinal dorsal horn. The DLPT includes the noradrenergic neurons, such as the locus coeruleus (LC), A5 and A7, which are major sources of noradrenergic projections to the dorsal horn. These descending inputs especially from the RVM exert bidirectional (facilitatory and inhibitory) influence on nociceptive transmission in the spinal dorsal horn [1-3]. In earlier studies, attention has been mainly focused on

the descending inhibitory influence. However, recently, it has also been known that the descending input from the RVM facilitates neuronal responses in the spinal dorsal horn and contributes to persistent pain and hyperalgesia [2–4]. Descending modulation is not a static process but exhibits dynamic changes in response to persistent noxious input following peripheral inflammation and nerve injury [5–7].

The mitogen-activated protein kinases (MAPKs) are a superfamily of intracellular signal transduction molecules that are evolutionally conserved [8, 9]. The MAPKs superfamily is made up of four main and distinct signaling pathways: the extracellular signal-regulated protein kinase1/2 (ERK1/2), the c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPKs), the p38 MAPKs, and the ERK5. Each of MAPKs signaling pathways involves a consecutive activation of four levels of signaling molecules: small GTPases (Ras or Rac), MAPK kinase kinases (Raf or MAPKKs), MAPK kinases (MEKs or MAPKKs), and MAPKs. The initial Ras and Rac localize to the inner surface of plasma membrane and transmit extracellular signals to

downstream components of MAPKs cascades (MAPKKKs). MAPKKKs activate MAPKKs, which are dual-specific kinases that phosphorylate at both Ser/Thr and Tyr sites, targeting a Thr-X-Tyr motif on the MAPKs (where X is glutamate (ERK1/2, ERK5), proline (JNK), or glycine (p38 MAPK)). The MAPKs, serine/threonine kinases, are activated by MAPKKs. Phosphorylation of the MAPKs results in a conformational change and a > 1000-fold increase in specific activity [10–12]. At the end of these signaling pathways, active MAPKs phosphorylate their target molecules, many of which are transcription factors, leading to facilitation of target gene expression. Thus, it is well established that neural MAPKs cascades play important roles in synaptic plasticity and remodeling during induction of long-term potentiation (LTP), learning, and memory consolidation [13, 14].

In the last 10 years, a number of studies have demonstrated that acute noxious stimuli, peripheral inflammation, and nerve injury activate MAPKs in several brain regions that are components of descending pain modulatory system [15-26]. These MAPKs activations play an important role in induction and maintenance of neural plasticity, which is thought to be essential for understanding the mechanism underlying dynamic changes in descending pain modulatory systems following peripheral tissue injury [5-7, 27]. To explore the role of each MAPK signaling pathway, the specific inhibitors such as ERK1/2 inhibitor (MEK inhibitor PD98059, U0126), p38 MAPK inhibitor (SB203580), and JNK inhibitor (SP600125) have been used in these studies [28]. The administrations of these inhibitors to descending pain modulatory systems alleviated hyperalgesia and allodynia in peripheral inflammatory pain models. [18, 21, 29]. In this paper, first, we introduce which MAPK is activated by such noxious stimuli and where those activations occur in descending pain modulatory system. Pain is a complex experience that involves not only the transduction of noxious environmental stimuli, but also cognitive and emotional processing in the brain [30]. Second, we discuss which of pain perception, pain-related emotional responses, and pain-related memory is the activation of MAPKs in these components related with it.

Stress affects brain activity and promotes long-term changes in multiple neural systems. A variety of environmental and/or stressful stimuli have been shown to induce not only pain suppression but also an increase in pain sensitivity. These phenomena are termed stress-induced analgesia (SIA) and stress-induced hyperalgesia (SIH), respectively [31]. Stress has also been found to exacerbate and could contribute to the etiology of chronic painful disorders, such as, fibromyalgia [32], irritable bowel syndrome [33], rheumatoid arthritis [34], and headache [35]. Psychophysical stress also activates MAPKs in brain structures related to descending pain modulatory system. MAPKs-induced neural plasticity in some of these structures might be associated with "limbically augmented pain syndrome" [36]. In this theory, stress and emotionally traumatic events lead to a sensitization of corticolimbic structures, which subserve both nociceptive processing and affective regulation. Therefore, we also discuss stress-induced activations of MAPKs in these structures.

2. Activation of Mitogen-Activated Protein Kinase in Descending Pain Modulatory System

2.1. Rostral Ventromedial Medulla (RVM). Peripheral inflammation induced by CFA injection into the hindpaw activated ERK1/2 and p38 MAPK in the RVM. The activation of ERK1/2 exhibited two characteristic phases. The first phase was a transient small increase at 30 minutes after CFA injection. The second phase was more persistent and pronounced increase from 3 hours to 24 hours, with a peak at 7 hours [15]. On the other hand, the activation of p38 MAPK was more short lived. It peaked at 30 minutes and lasted for 1 hour [16] (Figure 1). Phosphorylated ERK1/2 and p38 MAPK (p-ERK1/2 and p-p38 MAPK) were present predominantly in RVM neurons after CFA injection. About 60% of p-ERK1/2 neurons and 40% of p-p38 MAPK neurons in the RVM were serotonergic neurons [15, 16]. Microglial p-p38 MAPK in the RVM has also been reported to increase following carrageenan-induced inflammation [18].

Microinjection of U0126, an MEK inhibitor, into the RVM partially restored a decrease of paw withdrawal latency to noxious heat stimulus into the inflamed hindpaw [29]. ERK1/2 is involved in both transcription-independent and transcription-dependent forms of central sensitization. The former is early onset process, such as phosphorylation of receptors and trafficking of receptors to the synapse, and the latter is late onset, such as an increase in the expression of late-response genes [27, 37]. Since the microinjection of U0126 into the RVM significantly attenuated thermal hyperalgesia at 24 hours, but not at 7 hours after CFA injection [29], activation of ERK1/2 in the RVM might be involved in transcription-dependent plasticity. It has been demonstrated that the phosphorylation of ERK1/2 activates the transcription of tryptophan hydroxylase (TPH), the rate-limiting enzyme in serotonin biosynthesis, in the serotonergic neuron-like cell line [38]. Thus, activation of ERK1/2 in RVM serotonergic neurons is assumed to increase serotonin biosynthesis. 5-hydroxytryptamine (5HT) released from the descending bulbospinal neurons seems to exert dual (inhibitory and facilitatory) effects on spinal nociceptive processing [2, 3]. Oyama et al. [39] reported that the inhibitory and facilitatory effects were mediated by 5HT1A and 5HT3 receptors, respectively. Nearly onehalf of DRG neurons projecting to the superficial dorsal horn express 5HT3 receptor [40], and activation of 5HT3 receptor localized on central terminals of DRG neurons seems to enhance the release of neuropeptides [41]. Recently, by depleting endogenous 5HT in the RVM serotonergic neurons, it has been demonstrated that the RVM 5HT system participates in descending pain facilitation but not descending inhibition, which is necessary for maintenance of hyperalgesia and allodynia after peripheral inflammation and nerve injury [42]. Thus, it is possible that ERK activation induced by inflammation increases the transcription of TPH and serotonin biosynthesis, leading to the enhancement of hyperalgesia via descending serotonergic pathways.

Microinjection of SB203580, a p38 MAPK inhibitor, into the RVM attenuated carrageenan-induced thermal



FIGURE 1: (a) Time courses of p-ERK1/2 and p-p38 MAPK in the RVM after CFA injection into the hindpaw. (b) Photomicrographs showing p-ERK1/2- and p-p38 MAPK-immunoreactive neurons in the RVM (bregma -11.00 mm) following hindpaw inflammation. Scale bar = $100 \mu \text{m}$.

hyperalgesia and tactile allodynia [18]. The activation of p38 MAPK is involved in tumor necrosis factor- α and interleukin-1 production [43]. Since these cytokines phosphorylate NMDA receptor in the RVM neurons and cause allodynia [44], glial p38 MAPK may contribute to descending pain facilitation via cytokine production. In CA1 pyramidal

neurons, a small amount of Ca²⁺ ion influx via NMDA receptor activates the Rap-p38 MAPK signaling pathway, which drives the removal of synaptic AMPA receptors [45]. The activation of neuronal p38 MAPK in the RVM may contribute to a decrease in RVM excitability via the removal of synaptic AMPA receptors. AMPA receptor sensitivity in the RVM was reduced at 3 hours after the hindpaw inflammation, [46, 47]. Phosphorylation of p38 MAPK has also been shown to activate the transcription of TPH [38]. The activation of p38 MAPK in RVM serotonergic neurons may also contribute to serotonin biosynthesis. Thus, it is possible that p38 MAPK activation induced by inflammation increases glial cytokines and neuronal TPH production,

leading to the enhancement of hyperalgesia and allodynia. Chronic restraint stress (6 h daily for 3 weeks) induced thermal hyperalgesia and significant increase in activation of ERK1/2 in the RVM [48]. This stress-induced ERK1/2 activation in the RVM serotonergic neurons may also contribute to serotonin biosynthesis. The level of TPH in the RVM was significantly increased in the rats with chronic restraint stress [48]. Meanwhile, many studies have reported that chronic stresses decrease growth-associated and cytoskeletal proteins and induce neuronal atrophy in the hippocampus [49–51]. Since the sustained activation of ERK1/2 has been shown to be involved in neuronal degeneration [52], the stress-induced activation of ERK1/2 may be associated with neuronal atrophy and dendritic reorganization in the RVM.

2.2. Locus Coeruleus (LC). Acute noxious stimulation induced by formalin injection into the hindpaw activated ERK1/2 in the LC for 1 hour after the injection. However, CFA-evoked chronic inflammation did not induce a prolonged activation of ERK1/2 in the LC. After formalin injection, p-ERK1/2 was almost exclusively (more than 90%) located in the tyrosine hydroxylase- (TH-) positive neurons of the LC [17].

TH is the rate-limiting enzyme in NA biosynthesis. Short-term regulation of TH is accomplished by changes in the phosphorylation of this enzyme. ERK1/2 phosphorylates Ser₃₁ in TH [53, 54]. The phosphorylation of Ser₃₁ potentiates TH activity [55]. The activation of ERK1/2 in the LC after formalin injection might contribute to phosphorylation of TH and potentiation of TH activity. Furthermore, it has been shown that p-ERK1/2 activates c-fos, Fra-2, and CREB [56, 57]. The first two and the last interact with the AP-1 and CRE sites of the TH gene promoter, respectively [58]. Therefore, it has been speculated that activation of ERK1/2 in the LC increases TH gene transcription through the activation of Several transcription factors [59, 60]. The activation of ERK1/2 in the LC might increase TH gene transcription and NA biosynthesis.

Several studies have demonstrated that acute restraint stress increases p-ERK1/2 in the LC [17, 59–61]. On the other hand, chronic restraint stress (repeat 2–6 times) induced further marked activation of ERK1/2, JNK, and p38 MAPK in the LC [59]. Other study has reported that chronic restraint stress for 3 weeks decreases p-ERK1/2 in the LC [48]. The disparity among those experimental results may



FIGURE 2: Schematic drawing of noxious stimuli-induced MAPKs activations in the descending pain modulatory system. Boxes indicate noxious stimulation, activated MAPK, and function that is related to MAPK activation. PFC, prefrontal cortex; ACC, anterior cingulate cortex; AMY, amygdala; HPT, hypothalamus; PAG, periaqueductal gray; LC, locus coeruleus; RVM, rostral ventromedial medulla; DLF, dorsolateral funiculus; SDH, spinal dorsal horn; PAF, primary afferent fiber; POMC, proopiomelanocortin. Upward and downward arrowheads indicate increase and decrease, respectively.

be due to the differences in duration of restraint stress and experimental protocol. Short-term anaesthesia has been shown to induce ERK1/2 phosphorylation in the brainstem [62]. LC neurons in waking animals are very responsive to nonnoxious auditory, visual, and somatosensory stimuli in the environment [63–66]. Thus, we must take careful note of anesthesia, handling of animals, and experimental environment to evaluate an activation of MAPK in the LC.

2.3. Periaqueductal Grey (PAG). There are few studies that investigate activation of MAPK in the PAG. Some studies have reported the activation of ERK1/2 in the PAG after visceral noxious stimulation [19, 20]. Intraperitoneal injection of acetic acid significantly activated ERK1/2 in the PAG [19, 20]. The PAG has a longitudinal columnar organization, and each PAG column coordinates a distinct pattern of behavioral and physiological reactions critical for survival. The lateral and ventrolateral cell columns contain many



FIGURE 3: Schematic drawing of stress-induced MAPKs activations in the descending pain modulatory system. Boxes indicate psychophysical stress, activated MAPK, and function that is related to MAPK activation. For abbreviations see Figure 2.

neurons that project to the RVM [67]. Many p-ERK1/2 neurons were found in the lateral, ventrolateral, and dorsal columns. However, the densities of p-ERK1/2 neurons in these columns were not significantly different [20]. These results indicate that ERK1/2 is activated in several PAG neurons related to the different functional activity such as fear, anxiety, defensive reactions, and autonomic regulation in response to nociceptive stimuli.

2.4. Amygdala. The amygdala is now recognized as an important player in the emotional-affective dimension of pain [68, 69]. It has also been demonstrated that this

structure modulates nociceptive behavior by affecting the activity of RVM [70, 71]. Peripheral inflammation activated ERK1/2 in the amygdala at 3 h after formalin injection into the hindpaw. Formalin-induced p-ERK1/2 neurons were almost exclusively located in the laterocapsular division of the central nucleus of the amygdala (CeLC) [21]. It is noteworthy that ERK1/2 activation was seen in the right CeLC, independent of the side of peripheral inflammation. Inhibition of ERK1/2 activation in the amygdala by U0126 significantly decreased mechanical but not thermal hypersensitivity. Pharmacological activation of ERK1/2 in the amygdala induced mechanical hypersensitivity in the

absence of inflammation. These results have clearly shown that ERK1/2 activation in the amygdala plays a pivotal role in inflammation-induced mechanical hypersensitivity. ERK1/2 activation mediates plasticity in various brain regions. ERK1/2 activation in the CeLC neuron also contributes to synaptic facilitation by increasing NMDA receptor function after peripheral inflammation [72]. Furthermore, it has been demonstrated that ERK1/2 activation in the CeLC is downstream of metabotropic glutamate receptor 5 (mGluR5) [73]. The level of mGluR5 in the right amygdala was higher than that in the left amygdala. This seems to be one of the mechanisms for hemispheric lateralization of pain processing in the amygdala.

About the stress-induced activation of MAPKs, forced swim stress activated JNK, but not ERK1/2 and p38 MAPK, in the amygdala [74, 75]. The JNK signaling pathway is a major regulator for activation and expression of the AP-1 transcriptional factors such as c-Jun, c-Fos, and ATF. The activation of JNK may participate in stress-induced plastic change of the amygdaloid neurons. Maternal deprivation in early life increased immobility time in forced swim test and activation of ERK1/2 in the amygdala. Microinjection of PD98059 into the amygdala suppressed the immobility time. Thus, ERK1/2 activation in the amygdala seems to be implicated in the formation of depressive-like behavior [76].

2.5. Cerebral Cortex. The cerebral cortices including the anterior cingulate (ACC) and prefrontal cortices (PFCs) are believed to play important roles in emotion, learning, memory, and persistent pain in the adult brain [77–79]. The electrical stimulation of the ACC produces facilitation of tail-flick reflex induced by noxious heating via the RVM [80]. Formalin injection into the hindpaw activated ERK1/2 in rostral ACC (rACC) bilaterally [22]. A significant increase of ERK1/2 activation in the rACC occurred at 3 min, peaked at 10-30 min, and declined at 2 h but still remained after 24 h. Inhibition of ERK1/2 activation in the rACC by microinjection of PD98059 did not affect formalin-induced nociceptive behavior. However, PD98059 inhibited acquisition of formalin-induced conditioned place avoidance (F-CPA), which is believed to reflect pain-related negative emotion. ERK1/2 activation in the rACC required NMDA receptor, and it mediated CREB phosphorylation. However, the target genes regulated by CREB in the rACC remain to be elucidated. The activation of MAPKs including ERK1/2, JNK, and p38 MAPK has been shown to be critical for induction of LTP in the ACC [81]. The results have demonstrated that ERK1/2 activation in the rACC is critical for the development of affective pain (pain-related emotional response) but not nociceptive pain [22]. Peripheral nerve injury, such as digit amputation, also activated ERK1/2 in the ACC at 2 weeks after amputation [23]. Nonnoxious mechanical stimulation by brushing the hindpaw with amputated digit increased further the number of p-ERK1/2 neurons in the ACC and p-ERK1/2 in the dendrite and synaptic sites. The activation of ERK1/2 at the synaptic sites is thought to be involved in rapid synaptic potentiation and regulation of neuronal excitability [81, 82].

Acute stress such as restraint and forced swim activates ERK1/2 and JNK in the PFC and cingulate cortex [74, 83]. On the other hand, the effects of chronic stress on MAPKs activation in the PFC are inconsistent across the previous studies. Chronic restraint and forced swim stresses decreased ERK1/2, JNK, and p38 MAPK activations in the PFC [83-85]. In contrast, chronic stress induced by inescapable footshock increased ERK1/2 activation in the PFC [86, 87]. What is the functional role of stressinduced alteration of MAPK activity in the PFC? Singleprolonged stress (SPS) consists of restrain for 2 h, forced swim for 20 min and ether anesthesia, and exposure to SPS activated ERK1/2 in the mPFC. Inhibition of ERK1/2 activation in the mPFC ameliorated stress-induced anxietylike behavior, learning, and spatial memory impairment [88]. Antidepressant reversed stress-induced reduction of ERK1/2 activation in the PFC [85]. ERK1/2 activity in the PFC may be critical to depressive-like behavior and memory impairment. The rats subjected to prenatal stress showed a decrease of p38 MAPK activation in the PFC [89]. In these stressed rats, protein phosphatase-2A that dephosphorylates all MAPKs has been found to increase in the PFC. It has also been reported that p38 MAPK activation is involved in LTD at excitatory synapses of PFC pyramidal neurons [90]. These stress-induced reductions of MAPKs activation may impair synaptic plasticity. On the other hand, since sustained activation of MAPK induces neuronal degeneration [52], it is speculated that chronic stress-induced ERK1/2 activation may cause neuronal atrophy and reorganization [86, 87].

2.6. Hypothalamus. Electrical stimulation or opioid microinjection in the hypothalamus produces analgesia, which has been considered to play an important role in the modulation of pain. Beta-endorphin neurons in the hypothalamic arcuate nucleus (Arc) project to the PAG and activate descending projection neurons to the RVM in the PAG by inhibiting inhibitory GABA-ergic interneurons. This neural circuit has been implicated in the production of stimulation-produced and stress-induced analgesia [1, 2, 91]. Formalin injection into the hindpaw activated ERK1/2 in the hypothalamus [24]. ERK1/2 activation markedly increased at 30 min and remained higher than baseline after 24 h. p-ERK1/2 was colocalized with beta-endorphin in the Arc neurons. Proopiomelanocortin (POMC) is a precursor to several active peptides, including beta-endorphin. The i.c.v. injection of PD98059 attenuated formalin-induced increase of POMC mRNA expression in the hypothalamus. These results indicate that ERK1/2 activation in the hypothalamus may contribute to neuroendocrine regulation [24]. ERK1/2 activation in the hypothalamic paraventricular nucleus (PVN) has also been reported after intraplantar formalin or i.t. SP injections [25, 26]. The i.c.v. injection of PD98059 attenuated the second phase of formalin-induced nociceptive behavior [26]. Therefore, ERK1/2 activation in the PVN may be involved in acute nociceptive behavior.

Acute restraint stress activated ERK1/2 in the Arc and the PVN [60, 92]. Acute swim stress also activates JNK in the hypothalamus [75]. However, chronic restraint stress did not activate ERK1/2 in the Arc and the PVN [60]. These activations might be involved in autonomic and endocrine responses to the stress. Those functions remain elusive, however.

3. Conclusions and Perspectives

The noxious stimuli induced activations of MAPKs in the components of descending pain modulatory system. The activations in these components were associated with pain perception and pain-related emotional responses (Figure 2). In addition, psychophysical stress also activated MAPKs in these structures. They seem to be mainly related to depressive-like behavior (Figure 3). MAPKs are involved in both transcription-independent and transcription-dependent forms of central sensitization. Stress-induced neural plasticity in these structures via activations of MAPKs might affect nociceptive processing. In turn, the noxious stimuli-induced neural plasticity might potentiate depressive-like behavior in response to psychological stress. Clinical studies have demonstrated a reciprocal interaction between emotionality and pain perception in chronic pain conditions [93]. Elucidation of their physiological functions might contribute to a better understanding of chronic pain and lead to the development of new treatment to a variety of pain syndromes.

Recently, several studies have demonstrated that activation of ERK5 in the dorsal root ganglion and the spinal dorsal horn is related to hyperalgesia and allodynia following peripheral tissue injury [94–96]. Although there is no study that has examined the noxious stimuli-induced ERK5 activation in the supraspinal structures such as descending pain modulation system, one study has reported a reduction of ERK5 activity in the frontal cortex following psychophysical stress [97]. More importantly, PD98059 and U0126, specific inhibitors of MEK1/2-ERK1/2, also inhibit MEK5-ERK5 pathway [98]. Therefore, further studies are needed to examine whether the noxious stimuli induce ERK5 activation in descending pain modulation system and, if so, to explore what kinds of functions that activation is related to.

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Research Article

Repetitive Peroxide Exposure Reveals Pleiotropic Mitogen-Activated Protein Kinase Signaling Mechanisms

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Oxidative stressors such as hydrogen peroxide control the activation of many interconnected signaling systems and are implicated in neurodegenerative disease etiology. Application of hydrogen peroxide to PC12 cells activated multiple tyrosine kinases (c-Src, epidermal growth factor receptor (EGFR), and Pyk2) and the serine-threonine kinase ERK1/2. Peroxide-induced ERK1/2 activation was sensitive to intracellular calcium chelation and EGFR and c-Src kinase inhibition. Acute application and removal of peroxide allowed ERK1/2 activity levels to rapidly subside to basal serum-deprived levels. Using this protocol, we demonstrated that ERK1/2 activation tachyphylaxis developed upon repeated peroxide exposures. This tachyphylaxis was independent of c-Src/Pyk2 tyrosine phosphorylation but was associated with a progressive reduction of peroxide-induced EGFR from the plasma membrane to the cytoplasm. Our data indicates that components of peroxide-induced ERK1/2 cascades are differentially affected by repeated exposures, indicating that oxidative signaling may be contextually variable.

1. Introduction

Our current knowledge of the complexity of cellular signaling systems is only now allowing us to appreciate the true depth of connectivity in what was previously thought to be collections of linear transduction cascades. It is clear from many lines of study that the structure of signaling systems is far more complex than first thought [1–4]. Cellular signaling networks underpin most of the biological systems that help maintain physiological systems. Intracellular signal transduction, often referred to as intermediary metabolism, can be controlled by the activation of cell surface receptors, alterations in ion flux through specific or nonspecific transmembrane channels, and also by small chemical molecules that are produced through enzymatic or chemosynthetic processes such as nitric oxide or hydrogen peroxide [5]. Other chemically minimal agents such as hydroxyl radicals or peroxynitrite have also been studied, with the latter recently attracting attention as a potential signaling molecule [6]. With respect to the involvement of these simple signaling agents, both nitric oxide and hydrogen peroxide have garnered the most interest. Nitric oxide signaling mechanisms have been well delineated and are primarily connected with the modulation of guanylyl cyclase-dependent processes [7-10]. In contrast to nitric oxide, reactive oxygen species (ROS), such as hydrogen peroxide, are known to regulate a multitude of cellular signaling and physiological processes. The generic term, ROS, includes species such as hydrogen peroxide (H₂O₂), the hydroxyl radical (•OH), superoxide (O_2^{-}) , and singlet oxygen. ROS were originally recognized as active products produced from phagocytic neutrophils [11], which were thought to act as cytotoxic antimicrobial agents. A great deal of research upon ROS has therefore focused upon its association with tissue pathologies, for example, tumorigenesis and responses to hypoxia [12]. With respect to cancer signaling pathways, ROS are thought to contribute to aberrant cell growth through interference with multiple signaling systems involving nuclear transcription factor κ B, activated protein-1, phospholipase A₂, and mitogenactivated protein kinases (MAPKs) such as the extracellular signal-regulated kinase (ERK), Akt, and Jun kinase [12].

Hydrogen peroxide is the major physiologically relevant form of ROS due to its relatively high stability. Many nonphagocytic cells are now known to produce H₂O₂ in response to a variety of physiological stimuli such as cytokines, peptidergic growth factors and neurotransmitters [13-16]. Liberated H₂O₂, even at subtoxic concentrations, affects the function of various proteins including transcription factors, phospholipases, protein kinases and phosphatases, ion channels, G proteins, G protein-coupled receptors, and receptor tyrosine kinases [16-20]. One of the primary signaling actions that may associate both the physiological and pathophysiological actions of H_2O_2 is the control of cellular tyrosine phosphorylation. H₂O₂ plays a key role in reversible protein phosphorylation through modulation of protein-tyrosine phosphatases, the lipid phosphatase, and tumor suppressor PTEN as well as multiple receptor tyrosine kinases, potentially through direct oxidation of their catalytic cysteine residues [21–24]. H₂O₂ therefore has been recognized as an important signaling mediator in growth factor functionality and more specifically, cell signalingregulated phosphotyrosine posttranslational modification [23]. In many *in vitro* experiments, such as those mentioned previously, the signaling effects of H_2O_2 are usually explored in the context of persistent and chronic exposure to H₂O₂. As the generation and functioning of H₂O₂ may be controlled by humoral signaling systems in physiological settings, it is highly likely that, in vivo, the presence of H₂O₂ may vary from time to time according to physiological neurotransmission or hormonal release patterns. To investigate the effects of intermittent H₂O₂ exposure, we studied the effects of repetitive H₂O₂ exposure to neural PC12 cells with regards to cellular tyrosine kinase and ERK-activation properties. Both singular and repeated exposure of PC12 cells to H₂O₂ resulted in rapid increases in whole-cell protein tyrosine phosphorylation, specific phosphotyrosine content increases in receptor and nonreceptor tyrosine kinases and the potent activation of ERK. Repeated exposures to H_2O_2 , however, resulted in the differential tachyphylaxis of specific components of the peroxide-dependent ERKactivation mechanisms. Such evidence may suggest that, in vivo, a complex interplay between multiple H₂O₂-controlled signaling processes may underlie its multiple physiological activities.

2. Materials and Methods

2.1. Chemicals and Reagents. Cell culture grade hydrogen peroxide, monodansylcadaverine (MDC), methyl- β cyclodextrin (Me β C), sucrose, filipin, and the protein kinase A inhibitor H-89 were obtained from Sigma-Aldrich (St. Louis, MO). PP2 (non-receptor Src-family tyrosine kinase inhibitor), wortmannin (phosphoinositide 3-kinase inhibitor), BAPTA-AM (cell-permeable calcium chelator), PD98059 (MEK1/2 inhibitor) and AG1478 (epidermal growth factor receptor kinase inhibitor) were all obtained from EMD Bioscience (Gibbstown, NJ). Qproteome subcellular compartmentalization purification kits were obtained from Qiagen (Qiagen, Valencia, CA). The generic antiphosphotyrosine antisera, PY99, anti-epidermal growth factor (EGF), anti-actin, anti-catalase, non-phosphorylated ERK1/2, anti-Grb2, anti-epidermal growth factor receptor, anti-lamin A, anti-Tim23 and anti-annexin V antisera were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An agarose-preconjugated PY20 (antiphosphotyrosine) slurry was also obtained from Santa Cruz Biotechnology. Anti-phosphosite-specific anti-Pyk2 (antiphospho-Tyr402/Tyr881) and anti-c-Src (anti-phospho-Tyr418) were obtained from Invitrogen (Carlsbad, CA) and BD Bioscience respectively. Anti-active, (phosphor-)ERK1/2 antisera were obtained from Cell Signaling Technology (Danvers, MA). Non-phosphorylated anti-Pyk2 antisera were obtained from BD Bioscience.

2.2. Cell Culture. Rat pheochromacytoma cells (PC12), obtained from the American Type Tissue Culture Collection (http://www.atcc.org/), were maintained as previously described [3]. Briefly, PC12 cells were maintained in RPMI 1640 media supplemented with 10% horse serum, 5% fetal bovine serum, and 1% antibiotic/antimycotic (penicillin/streptomycin/fungizone: Invitrogen, Carlsbad CA) at 37°C in a 5% CO₂ atmosphere. Prior to any cellular challenges with hydrogen peroxide, the cells were serum deprived for 16 h by removing the horse and bovine serum content of the RPMI growth medium and addition of 10 mM HEPES to compensate for the reduced buffering capacity caused by serum withdrawal. All PC12 cells were maintained in 100-mm plates coated with poly-d-lysine (Sigma Aldrich, St. Louis, MO). To experimentally degrade the exogenously-applied hydrogen peroxide, bovine liver catalase (Sigma Aldrich, St. Louis, MO) was added to the experimental media at a concentration of 40 U/mL. Bovine liver catalase was inactivated with a 95°C incubation for 10 minutes.

2.3. Immunoprecipitation and Western Blotting. After challenges with hydrogen peroxide, PC12 cell monolayers were placed on ice, washed twice in ice-cold Dulbecco's phosphate-buffered saline, and lysed in a Nonidet P-40 (NP40-)based solubilization buffer, as described previously [25]. Solubilized lysates were clarified by centrifugation at 15,000 rpm for 15 min and diluted to an approximate concentration of 1 mg/mL total protein. Subsequently, a 50 μ L aliquot of clarified whole-cell lysate was mixed with an equal volume of 2× Laemmli sample buffer and resolved by SDS-PAGE for determination of intracellular protein activation by immunoblotting. Immunoprecipitation (IP) of generic phosphotyrosine proteins was achieved

using 20 µL of a 50% slurry of anti-PY20 affinity agarose (Santa Cruz Biotechnology) with agitation for 16h at 4°C. Immunoprecipitation of Pyk2 or EGFR was achieved using the addition of $5 \mu g$ of the specific anti-protein sera plus 30 µL of a protein-A/G-conjugated agarose slurry (EMD Bioscience) followed by agitation for 16 h at 4°C. Immune complexes were washed three times with icecold NP40-based lysis buffer and transferred to a clean microcentrifuge tube before addition of 20 μ L of 2× Laemmli sample buffer. Immunoprecipitates were resolved by SDS-PAGE and electrotransferred to polyvinylidene difluoride (PerkinElmer Life Sciences: Waltham, MA) membrane for protein immunoblotting (IB). Polyvinylidene difluoride membranes were blocked in a bovine serum albumin-based solution (4% bovine serum albumin, 50 mM Tris-HCl, pH 7.0, 0.05% Tween 20, 0.05% NP40) solution for 1 h at 37°C before immunoblotting. Immunoblotting of wholecell lysates for unphosphorylated/phosphorylated ERK1/2 or inactive/active c-Src (Tyr-418 autophosphorylated) was performed using specific primary antisera (see under "Chemicals and Reagents") at a 1:1000 dilution with subsequent addition of a 1:7000 dilution of alkaline phosphataseconjugated anti-mouse or rabbit IgG as a secondary antibody (Sigma Aldrich). Immunoblotting of anti-phosphotyrosine immunoprecipitates, resolved with SDS-PAGE, for phosphotyrosine content was performed using a 1:1000 dilution of PY99 antisera with a subsequent addition of a 1:7000 dilution of alkaline phosphatase-conjugated anti-mouse IgG as a secondary antibody (Sigma). Immunoblotting of anti-Pyk2 or EGFR immunoprecipitates was performed using either anti-PY20 (1:1000 dilution for primary followed by 1:7000 dilution of alkaline phosphatase-conjugated antimouse secondary antisera), anti-phosphosite-specific (Tyr-402 or Tyr-881) anti-Pyk2 antisera (1:1000 dilution followed by incubation with a 1:7000 dilution of an alkaline phosphatase-conjugated anti-mouse secondary antisera) or with anti-Grb2 antisera (1:1000 primary antisera dilution followed by a 1:7000 dilution of alkaline phosphatase antirabbit-conjugated secondary antisera). Specifically isolated Qproteome cell compartments (CE1-cytoplasmic; CE-2 plasma membrane; CE-3 nuclear) were identified using 1:1000 dilutions of specific primary antisera, CE1-annexin-V, CE2-Tim23, or CE3-lamin-A followed by 1:7000 dilutions of species-specific alkaline phosphatase-conjugated secondary antisera (lamin A-mouse; Tim23-goat; annexin V-mouse). Visualization of alkaline phosphatase-labeled proteins was performed using enzyme-linked chemifluorescence (GE Healthcare-Amersham Biosciences) and quantified using a Typhoon 9410 PhosphorImager (GE Healthcare). Image density measurements were performed from phosphorimager-captured images using L-Process version 2.2 and Image-Gauge version 4.2 (Fuji Image Systems). Western blot image density was measured per square visual pixel (px^2) as arbitrary units (AUs) from which background (B) signals were subtracted. Therefore western image density is represented throughout as (AU-B)/px². Statistical analysis of relative protein expression values was performed using an unpaired Student's t-test in GraphPad Prism version 5.0.

3. Results

3.1. Hydrogen Peroxide Exposure Induces Widespread Changes in Generic Cellular Protein Tyrosine Phosphorylation Status. Short term application (10 minutes) of hydrogen peroxide to serum-deprived PC12 cells resulted in the dose-dependent increase in the generic phosphotyrosine content of multiple proteins identified using poylacrylamide gel resolution of anti-phosphotyrosine (PY20) immunoprecipitates derived from cell lysates. The dose response relationship, however, was not perfectly sigmoidal, as at peroxide concentrations greater than $200 \,\mu\text{M}$ a lack of further phosphotyrosine increase was seen (Figure 1(a)). Employing the hydrogen peroxide concentration that yielded the most robust increases in whole-cell protein phosphotyrosine content $(100 \,\mu\text{M})$, it was demonstrated that this effect was achieved and reached its maximum after 10 minutes of acute stimulation (Figure 1(b)). This time period of acute stimulation was therefore employed as the standard stimulation time in all subsequent experiments.

3.2. Acute Hydrogen Peroxide Exposure Induces a Dose-Dependent Increase of the Phosphorylation Status of Serine/Threonine and Tyrosine Kinases. Acute (10 minute) application of multiple doses $(10-100 \,\mu\text{M})$ of hydrogen peroxide resulted in the significant increase of the levels of tyrosine-418 phosphorylation of the non-receptor tyrosine kinase c-Src (Figures 1(c) and 1(d)). This tyrosine phosphorylation site on c-Src occurs in the kinase activation region of the Src family kinases and is highly indicative of auto-tyrosine phosphorylation mediated by the intrinsic Src kinase activity itself. Significant dose-dependent increases in the phosphotyrosine status of the immunoprecipitated epidermal growth factor receptor (EGFR) were also noted in response to the acute hydrogen peroxide stimulation (Figures 1(c) and 1(e)). In an analogous manner to c-Src, upon activation EGFRs typically auto-tyrosine phosphorylate at multiple residues. This phosphorylation event is highly indicative of activation of the EGFR intrinsic tyrosine kinase. To assess whether any residual EGF (from initial serum-containing media used for prior cell growth) was present during the peroxide exposure, specific Western blot analysis was performed on nonconcentrated and SpeedVac-concentrated serum-deprivation media. We found that no extracellular EGF ligand was present in the serum-deprivation media during the peroxide exposures (data not shown). In addition to increases in the phosphotyrosine content of c-Src and the EGFR, we also noted a significant peroxide dose-dependent increase in the phosphotyrosine content of Pyk2, isolated using specific anti-Pyk2 immunopurification (Figures 1(c) and 1(f)). It has been demonstrated that all of these tyrosine kinase systems, c-Src, EGFR, and Pyk2, are similarly linked to conserved cellular signaling mechanisms that directly control the activation of MAPKs such as the serine/threonine targeting extracellular signal-regulated kinase (ERK1/2). When we assessed the activity status of ERK1/2 in response to the peroxide exposure, we noted a significant elevation in the activity status of ERK1/2 (Figures 1(c) and 1(d)) that coincided with the peroxide-induced



FIGURE 1: Hydrogen peroxide exposure generates increases in tyrosine phosphorylation status and activation of multiple signaling proteins in PC12 cells. (a) Hydrogen peroxide dose-dependent (10–1000 μ M) increases in whole-cell protein tyrosine phosphorylation. Hydrogen peroxide exposure time was 10 minutes. Total tyrosine phosphoproteins were purified by immunoprecipitation (IP) from PC12 cells using anti-phosphotyrosine antisera (PY20). Immunoblotting (IB) detection of the phosphotyrosine was achieved with an antisera raised against a differential phosphotyrosine immunogen (PY99). The associated histogram depicts the whole-lane image tyrosine phosphoprotein density quantitation measured as AU-B/px². (b) Time-dependent hydrogen peroxide (100 μ M) increases in whole-cell protein tyrosine phosphorylation. The associated histogram depicts the whole-lane image tyrosine phosphoprotein density quantitation measured as AU-B/px². (c) Hydrogen peroxide (10 minutes) exposure induces the activation of extracellular signal-regulated kinase (ERK1/2: detected in 2% of the total protein from a whole-cell (w.c.) lysate), tyrosine phosphorylation of Pyk2 and the epidermal growth factor receptor (EGFR) and activation of the non-receptor tyrosine kinase c-Src. (d), (e), (f), and (g) depict the peroxide-induced fold changes in phosphorylation of c-Src, EGFR, Pyk2, and ERK1/2, respectively. Values in each histogram depict the mean ± standard error for three individual experiments for each set of bars. For statistical analysis probability values indicated are **P* < .05, ***P* < .01, and ****P* < .001.

increases in phosphotyrosine content of Pyk2 and the EGFR as well as the activity status of c-Src.

3.3. Acute Removal of Applied Hydrogen Peroxide Attenuates Long-Term ERK Activation in PC12 Cells. As we noted that acute application of hydrogen peroxide to PC12 cells resulted in the simultaneous activation of multiple tyrosine kinase systems as well as ERK1/2, we investigated the ability of the PC12 cells to recover from such acute exposures. During neurological processes in aging, or during periods of stress, it is possible that cellular damage may be induced by both intermittent as well as chronic and protracted oxidative episodes. Exposure to long-term stresses tends to induce pathophysiologies and even cell death mechanisms whereas short-term exposures are more survivable [26, 27]. Application of $100 \,\mu$ M hydrogen peroxide for at least 60

minutes resulted in a long-lasting elevation of the ERK1/2 activity status in the PC12 cells (Figure 2(a)). To simulate a short-term, survivable exposure to the peroxide, we performed parallel experiments to compare peroxide exposure paradigms that involved rapid acute exposure versus longterm exposure to the oxidative agent. Using multiple parallel experiments, we were able to simultaneously generate PC12 protein extracts at any time point during the experiment. We therefore applied the $100 \,\mu\text{M}$ hydrogen peroxide dose for 10 minutes and then removed the peroxide-containing media and replaced it with media similar to that prior to the peroxide exposure (Figure 2(b)) to achieve an "acuterecovery" process. PC12 cell lysates were then extracted from plates that were naive to peroxide, directly at the end of the 10 minute peroxide exposure and then 10, 30, and 60 minutes after the removal of the peroxide exposure (recovery) (Figure 2(b)). We additionally performed control experiments to assess any effects of fluid replacement upon ERK1/2 activity in the PC12 cells. When performing multiple fluid exchanges, we did not notice any significant alterations in the PC12 ERK1/2 activity status in our cells (data not shown). We noted that the degree of ERK1/2 activation after the "acute-recovery" peroxide paradigm waned rapidly to nearly background levels at 30 minutes after the "acuterecovery" peroxide stimulation (Figure 2(c)). Therefore after 30 minutes from the removal of the acutely applied peroxide, the ERK1/2 activity reached basal levels, while with sustained peroxide exposure the PC12 cells demonstrated a well-maintained high-activity status of ERK1/2. Using this "acute-recovery" peroxide stimulation procedure, that is, 10minute peroxide application followed by peroxide removal and a 10 minute recovery before protein harvesting, we also assessed the changes in activity status of c-Src, Pyk2, and EGFR. In a similar manner to the standard peroxide application, without removal and recovery, we noted that the "acute-recovery" peroxide protocol was capable of inducing significant increases in the phosphorylation of c-Src Tyr-418 resolved from whole-cell lysates (Figure 2(d)). In addition, phosphotyrosine immunoblotting of Pyk2 (Figure 2(e)) or EGFR (Figure 2(f)) immunoprecipitates demonstrated that the "acute-recovery" peroxide paradigm induced significant elevations of the phosphotyrosine content of these tyrosine kinases. In addition to assessment of the signaling functionality of the "acute-recovery" peroxide exposure, we demonstrated that the primary activity of the peroxide was mediated through its extracellular effects, rather than intracellular permeation, with the application of exogenous catalase (Figures 2(g) and 2(h)). After addition of active bovine liver catalase (40 U/mL) to the cell media (30 minutes preincubation) prior to peroxide exposure, we noted a significant reduction in the ERK1/2 activation signal. Heat treatment (95°C, 10 minutes) of the catalase before its addition to the peroxide-containing media prevented the inhibition of ERK1/2 activation (Figures 2(g) and 2(h)). Specific Western blot analysis of whole-cell lysates incubated for 30 minutes with extracellular catalase failed to demonstrate any significant changes of intracellular catalase levels, suggesting that negligible catalase internalization had occurred (Figure 2(i)). Therefore, the majority of the rapid

effects of catalase upon peroxide activity are likely to have occurred in the extracellular space.

3.4. Chemical Sensitivity of "Acute-Recovery" Peroxide-Induced ERK Activation. We next examined the potential mechanistic links between the tyrosine phosphorylation events induced by the "acute-recovery" peroxide exposure process and the ERK1/2 activation. Preincubation of PC12 cells with the specific EGFR kinase inhibitor AG1478, the c-Src kinase inhibitor PP2, and the Ca²⁺-chelator BAPTA-AM induced a partial inhibition of the ERK1/2 signal (Figures 3(a) and 3(b)). The inhibitory effects of BAPTA-AM were likely due to the chelation of extant intracellular calcium as our low peroxide dose employed (100 μ M) did not greatly elevate global calcium levels (data not shown). Direct inhibition of the upstream ERK1/2-phosphorylating kinase, MEK1/2, with PD98059 ($20 \mu M$, 60 minute preincubation prior to peroxide exposure) resulted in near complete inhibition of the peroxide-induced ERK1/2 signal. The peroxide-induced ERK1/2 activation signal was, however, largely resistant to inhibition of phosphoinositide-3-kinase (PI-3K) or protein kinase A (PKA) as neither H-89 nor wortmannin preincubation affected the peroxide-induced ERK1/2 activation (Figures 3(a) and 3(b)).

3.5. Acute-Recovery Peroxide Exposure Protocols Allow Assessment of Repeated Peroxide Exposure-Induced ERK1/2 Tachyphylaxis. As the "acute-recovery" peroxide exposure paradigm allows the peroxide-induced ERK1/2 response to subside to basal levels within thirty minutes after peroxide removal, we performed multiple parallel experiments to analyze the ability of the PC12 cells to respond to multiple acute exposures. The pictogram in Figure 3(c) demonstrates the methodology of investigating the ability of the PC12 cells to respond to four successive peroxide exposures (all $100 \,\mu\text{M}$ doses). To create protein samples for R1, the simple "acuterecovery" process was performed. To create protein samples for secondary (R2), tertiary (R3), and quaternary (R4) responses, individual "acute-recovery" peroxide applications were made and were separated by 30 minutes of recovery time (Figure 3(c)). When the levels of peroxide-induced ERK1/2 activation were measured for R1 to R4 responses it was noted that the degree of peroxide-induced ERK1/2 activation was significantly reduced by R3 and then again for R4. The greatest reduction in peroxide-induced ERK1/2 signal was observed for the R4 response, compared to R1. There was no significant diminution of the peroxideinduced ERK1/2 activation from R1 to R2 (Figure 3(d)). As the biggest changes in peroxide-induced ERK1/2 activation occurred at R4, we directly compared potential signaling mechanism differences between stimulation protocols for R1 samples compared to R4 samples.

3.6. Repeated Peroxide Exposure Causes Reduced EGFR Tyrosine Phosphorylation but Fails to Significantly Affect Pyk2 and c-Src Tyrosine Phosphorylation. As we have shown in the previous section, repeated peroxide "acute-recovery" exposures induce a partial tachyphylaxis of the ERK1/2



FIGURE 2: ERK1/2 responses to chronic or acute-recovery exposure to hydrogen peroxide. (a) Chronic hydrogen peroxide exposure (70minutes, 100μ M) and ERK1/2 activation (white bar-no peroxide, grey bar-peroxide exposure). (b) Acute-recovery hydrogen peroxide exposure (100μ M) and ERK1/2 activation. (c) Chronic (black bars) versus acute-recovery (grey bars) peroxide exposure ERK1/2 activation profile. Acute-recovery peroxide exposure-induced activation of c-Src (d), Pyk2 (e) and EGFR (f) tyrosine phosphorylation. (g)-(h) Catalase (40 U/mL) (active or heat-inactivated) effects upon peroxide-induced (acute-recovery protocol) ERK1/2 activation. (i) Intracellular catalase levels following extracellular enzyme exposure.



FIGURE 3: Chemical sensitivity and tachyphylaxis of the ERK activation response to the "acute-recovery" paradigm of hydrogen peroxide exposure. (a) Representative western blot of the chemical sensitivity of the acute-recovery paradigm peroxide-induced ERK activation (10 minute peroxide exposure, 10 minute recovery). Signaling reagents were pre-incubated with the PC12 cells prior to the "acute-recovery" peroxide exposure as follows: AG1478, 100 nM, 30 minutes; PD98059, $20 \,\mu$ M, 60 minutes; BAPTA-AM, $50 \,\mu$ M, 30 minutes; wortmannin, 10 nM, 30 minutes; PP2, $5 \,\mu$ M, 30 minutes; H-89, $10 \,\mu$ M, 30 minutes. (b) Histogram depicting the relative effects of the chemical pre-exposures from panel A to the fold over basal-induced ERK1/2 activation induced by the acute-recovery peroxide protocol. Each histogram bar depicts the mean \pm standard error. (c) Diagrammatic representation of peroxide exposure procedures employed to derive protein extracts for examination of repeated peroxide exposure (grey panels) effects upon ERK1/2 activation (NS1—non-stimulated control ERK1/2 sample, R1—sample from 10 minutes after 10 minute acute-recovery peroxide exposure protein samples). (d) The ERK1/2 activation responses to the repeated acute-recovery peroxide exposure demonstrates tachyphylaxis. The acute-recovery R1 response (10 minute exposure, 10 minute recovery) was followed by a 30 minute recovery before the same acute-recovery process was repeated, generating the R2 response. R3 and R4 responses were created in a similar manner from cells previously stimulated with R1, R2 and then subsequent R3 and R4 acute-recovery exposures. The associated histogram represents the mean \pm standard error for three individual ERK1/2 R1-R4 tachyphylaxis experiments. For statistical analysis probability values indicated are **P* < .05, ***P* < .01.

response. We then investigated the effects of repeated peroxide exposure upon peroxide-induced c-Src activation as well as peroxide-induced EGFR and Pyk2 tyrosine phosphorylation. Upon comparing the effects of repeated exposure

to peroxide (R1 to R4), we noted that along with the expected reduction in ERK1/2 activation, at the R4 response point, the ability of the hydrogen peroxide to induce Pyk2 auto-tyrosine phosphorylation (indicative of Pyk2 tyrosine

kinase activation-measured with anti-phospho-Tyr-402-Pyk2 sera) and increase generic Pyk2 tyrosine phosphorylation was not significantly affected (Figures 4(a) and 4(b)). As with the Pyk2 responses between R1 and R4 peroxide exposures, the degree of c-Src Tyr-418 phosphorylation was not significantly diminished by the repeated exposure (R1-R4) to the stressor (Figures 4(a) and 4(b)). In contrast, however, we noted that the degree of peroxide-induced EGFR tyrosine phosphorylation was significantly lower at the R4 response point compared to R1 (Figures 4(a) and 4(b)). We additionally assessed whether there were any differences in the signaling pathways to ERK1/2 used by the peroxide exposures at either R1 or R4. When determining the R1 effects we again noted that the peroxide-induced ERK1/2 response was partially sensitive to AG1478, BAPTA-AM, and PP2 preincubation (Figure 4(c)). When this R1-sensitivity was compared to the R4 response chemical sensitivity, we noted that the R4-mediated ERK1/2 responses were relatively insensitive to AG1478-mediated inhibition of the EGFR intrinsic tyrosine kinase (Figure 4(d)). The R4 responses, however, were still sensitive to the preincubation with BAPTA-AM and PP2 (Figure 4(d)).

3.7. Repeated Peroxide Exposure Attenuates EGFR Tyrosine Phosphorylation and Protein-Protein Interaction with Growth Factor Receptor Binding Protein-2. As we had noted that repeated exposures to peroxide reduced both the ERK1/2 activation and the degree of EGFR tyrosine phosphorylation, but not that of Pyk2 or c-Src, we decided to investigate whether additional signaling components of ERK1/2activating complexes were affected. The growth factor receptor binding protein 2 (Grb2) is a bifunctional adapter protein involved in multiple ERK-activating multiprotein complexes. Grb2 is typically associated with Ras-guanine nucleotide exchange factors such as son of sevenless (Sos). Upon activation of scaffolding molecules employed in ERK1/2 activation cascades, for example, EGFR, Pyk2, or the focal adhesion kinase (FAK), the auto-tyrosine phosphorylation of these proteins attracts Grb2 to the plasma membrane via interaction of its SH2 domains with phosphotyrosine residues on the scaffolds. Grb2 interacts with Sos via its SH3 domains associating with proline-rich regions of Sos. At the plasma membrane, Sos can then facilitate exchange of guanosine triphosphate for guanosine diphosphate at juxtamembrane monomeric G proteins such as Ras. As Grb2 association with scaffolds, such as EGFR or Pyk2, is vital for their capacity to activate ERK1/2 cascades, we investigated the relative effects of R1- versus R4-level peroxide exposures upon Grb2 interaction with EGFR or Pyk2. As we have seen previously, successive peroxide exposures reduced the degree of peroxide-induced EGFR tyrosine phosphorylation (Figures 5(a) and 5(b)). When the Grb2 content of the EGFR immunoprecipitates was assessed, we noted that for R1 responses to peroxide exposure there was a significant increase in the Grb2 content of the purified EGFR (Figures 5(a) and 5(c)). With repeated peroxide exposures (R4), the degree of peroxide-induced EGFR tyrosine phosphorylation was attenuated (Figures 5(a) and 5(d)) along

with a significantly reduced Grb2 content of the EGFR immunoprecipitates (Figures 5(a) and 5(e)). We have shown previously that peroxide stimulation increases the generic and Tyr-402-specific tyrosine phosphorylation of Pyk2 at R1 and R4 response points (Figure 4(a)). With respect to association with Grb2, phosphorylation of the Pyk2 tyrosine residue 881 has been shown to be critical [28]. Using specific antisera that recognizes tyrosine phosphorylation of residue Tyr-881 on Pyk2, we noted that at the R1 and R4 time points peroxide increased the phosphotyrosine content of this residue (Figure 5(f)). At the R1 response point, peroxide exposure induced a significant increase in Pyk2 Tyr-881 phosphorylation status (Figures 5(f) and 5(g)) along with a significant increase in the Grb2 content of the Pyk2 immunoprecipitate (Figures 5(f) and 5(h)). Unlike the tyrosine phosphorylation and Grb2 interaction with the EGFR, repeated peroxide exposure (at R4 response point) did not significantly affect Pyk2 Tyr-881 phosphorylation (Figures 5(f) and 5(i)) or the Grb2 content of the Pyk2 immunoprecipitates (Figures 5(f) and 5(j)). While Grb2 was observed to increase in its association with either EGFR or Pyk2 upon peroxide exposure, we also tested the possibility that a multimolecular complex containing all three proteins may also be induced. When EGFR immunoprecipitates were probed for the presence of Pyk2 (in the presence or absence of peroxide exposure), we failed to find any significant coassociation of these two proteins. When the converse western blot was performed, no significant EGFR content of Pyk2 immunoprecipitates was also demonstrated in the absence or presence of peroxide (data not shown). Therefore, it seems likely that peroxide treatment induces the creation of largely discrete Grb2-interacting EGFR or Pyk2 complexes.

3.8. Repeated Peroxide Exposure Induces a Selective Subcellular Redistribution of ERK1/2-Activating Scaffold Proteins. It has been demonstrated by many researchers that the molecular and biochemical nature of a specific ERK1/2-activating scaffold has profound effects upon the physiological mechanisms of ERK1/2 signaling [4, 25]. To this end, we sought to investigate a potential difference in the ERK1/2-activating scaffolds in our study, EGFR and Pyk2, with respect to their abilities to respond to repeated peroxide exposure. The ability of many G protein-coupled receptors (GPCRs) to activate ERK1/2 has been shown to involve a productive engagement with classical endocytic processes [4]. This interaction has resulted in the demonstration of endocyticdependent ERK1/2-activation processes. Rather than residing with the importance of endocytosis of the GPCR, it appears in many cases that the specific endocytic process interaction of the scaffold/Ras-activating machinery is more important. We therefore assessed whether the inhibitory actions of repeated peroxide exposure upon EGFR-associated ERK1/2 signaling may be related to a subcellular redistribution of the EGFR compared to Pyk2. Using a differential detergent-based cell compartmentalization process (Qproteome), we were able to successfully isolate subcellular fractions that demonstrated enrichment of nuclear proteins (lamin A-CE3), plasma membrane proteins (Tim23-CE2),


FIGURE 4: Repeated peroxide exposure differentially affects tyrosine kinase responses in PC12 cells. (a) Comparison of levels of peroxideinduced ERK1/2 activation, Pyk2 generic tyrosine and tyrosine-402 phosphorylation, c-Src tyrosine-418 phosphorylation and EGFR tyrosine phosphorylation between R1 and R4 responses. (b) The histogram depicts the quantitation of mean \pm standard error for three individual experiments for the data represented in panel (a). The represented fold changes in ERK1/2, Pyk2 Tyr-402, Pyk2 generic tyrosine phosphorylation, c-Src Tyr-418 and EGFR tyrosine phosphorylation were calculated relative to the non-stimulated phosphorylation value prior to either R1 (black bars) or R4 (grey bars). (c) Chemical sensitivity of R1 versus R4 ERK1/2 phosphorylation responses to acuterecovery peroxide exposure paradigms. Preincubation times and chemical concentrations for AG1478, BAPTA-AM and PP2 were as used before in Figure 3. (d) The histogram depicts the quantitation of the mean \pm standard error for three individual experiments for the data represented in panel (c) (R1-black bars; R4-grey bars). For statistical analysis probability values indicated are **P* < .05, ***P* < .01.



FIGURE 5: Grb2 association with EGFR or Pyk2 is differentially affected by repeated peroxide exposure. (a) Representative western blots of phosphotyrosine and Grb2 content of EGFR immunoprecipitates from cells stimulated to R1 or the R4 level with 100 μ M hydrogen peroxide. (b) EGFR immunoprecipitate tyrosine phosphorylation status in response to R1 hydrogen peroxide exposure (NS—non-stimulated). (c) Peroxide-induced fold changes in the Grb2 content of EGFR immunoprecipitates at the R1 response point. (d) EGFR immunoprecipitate tyrosine phosphorylation status in response to R4 hydrogen peroxide exposure (PS—pre-stimulated basal level prior to R4). (e) Peroxide-induced fold changes in the Grb2 content of EGFR immunoprecipitates at the R4 response point. (f) Representative western blots of Pyk2 Tyrosine 881 phosphorylation and Grb2 content of Pyk2 immunoprecipitates from cells stimulated to R1 or the R4 level with 100 μ M hydrogen peroxide. (g) Pyk2 immunoprecipitate tyrosine-881 phosphorylation status in response to R4 hydrogen peroxide exposure. (j) Peroxide-induced fold changes in the Grb2 content of Pyk2 immunoprecipitates at the R1 response point. (i) Pyk2 immunoprecipitate tyrosine-881 phosphorylation status in response to R4 hydrogen peroxide exposure. (j) Peroxide-induced fold changes in the Grb2 content of Pyk2 immunoprecipitates at the R1 response point. (i) Pyk2 immunoprecipitate tyrosine-881 phosphorylation status in response to R4 hydrogen peroxide exposure. (j) Peroxide-induced fold changes in the Grb2 content of Pyk2 immunoprecipitates at the R1 response point. (i) Pyk2 immunoprecipitate tyrosine-881 phosphorylation status in response to R4 hydrogen peroxide exposure. (j) Peroxide-induced fold changes in the Grb2 content of Pyk2 immunoprecipitates at the R4 response point. The histograms in each panel depict the quantitation of the mean ± standard error for three individual experiments. For statistical analysis **P* < .05, ***P* < .01.

and cytoplasmic proteins (annexin V-CE1) (Figures 6(a)– 6(d)). Stimulation of cell surface receptors that are able to engage classical endocytic pathways often result in a rapid (10–30 minutes) redistribution from the plasma membrane to small early endocytic vesicles in the cytoplasm. When we applied successive peroxide exposures (R1–R4) and then isolated either the cytoplasmic (CE1) or plasma membrane (CE2) subcellular fractions, we noted that there was little alterations in the expression profile of Pyk2 across R1-R4 (Figures 6(e), 6(f), and 6(g)). In contrast, with successive peroxide exposures (R1–R4), we noted a significant increase in the amount of EGFR found in cytoplasmic extracts (Figures 6(e) and 6(h)), while a concomitant significant decrease of the EGFR found in plasma membrane extracts was observed (Figures 6(e) and 6(i)). Therefore it appeared that while Pyk2 expression levels did not significantly alter in the two compartments, there seemed to be a progressive relocalization of EGFR from the plasma membrane to the cytoplasm after repeated peroxide exposures. We additionally contrasted the nature of the peroxide-induced EGFR cellular



FIGURE 6: Peroxide-mediated subcellular relocalization of EGFR. (a) Subcellular fractionation verification (nuclear, lamin-A: CE3; plasma membrane, Tim23: CE2; cytosol, annexin-V: CE1) and quantitation ((b)-lamin-A, (c)-Tim23, (d)-annexin-V). (e) Peroxide effects (responses R1-R4) on Pyk2 and EGFR subcellular localization Relative expression profiles for Pyk2 (CE1-CE2 quantification (f)-(g)) and EGFR (CE1-CE2 quantification (h)-(i)). Chemical inhibitor-mediated alteration of R4 peroxide-mediated (j) or EGF (k) induced EGFR redistribution from CE2 fraction. EGF (10 ng/mL)-mediated redistribution was compared to non-stimulated (NS) cells.

redistribution process from the CE2 fraction using chemical inhibitors of two different forms of endocytic mechanisms, that is, clathrin or caveolae mediated [29]. Inhibition of clathrin-mediated endocytosis (30-minute preincubation before R4 peroxide exposure with $400 \,\mu\text{M}$ MDC or hypertonic 0.45 M sucrose) significantly attenuated the peroxideinduced EGFR redistribution (Figure 6(j)). Inhibition of caveolae-mediated endocytic mechanisms (30-minute preincubation with Me β C (10 μ M) or filipin (5 μ g/mL)) failed to alter R4 peroxide-induced EGFR redistribution (Figure 6(j)). When the chemical sensitivity of EGF-induced (10 ng/mL, 30 minute exposure) redistribution of EGFR from CE2 was analogized to that mediated by peroxide, we noted a similar inhibitory effect of clathrin-mediated endocytosis disruptors (MDC, sucrose) and relative independence from caveolae-mediated mechanisms (Figure 6(k)). These data suggest therefore that the peroxide-induced redistribution of EGFR occurs through a qualitatively similar process to that of EGF-induced EGFR internalization. In a similar manner to attenuation of ERK1/2 signaling upon repeated peroxide exposure, we also observed a similar progressive reduction in EGF-induced ERK1/2 phosphorylation (% ERK1/2 phosphorylation compared to non-stimulated control: EGF exposure 1, 20.5 \pm 1.3%; EGF exposure 2, 5.7 \pm 0.9%; EGF exposure 3, $1.19 \pm 1.03\%$; EGF exposure 4, $1.06 \pm 0.87\%$ (n = 3)) using a repeated exposure paradigm analogous to the peroxide stimulations. As both repeated peroxide and EGF mediated an effective redistribution of EGFR from the plasma membrane fraction, we also found that exogenous EGF application (10 ng/mL, 30 minutes) was able to partially blunt the subsequent ERK1/2 response to peroxide stimulation (% peroxide-induced ERK1/2 phosphorylation after EGF treatment, compared to non-EGF pre-exposed control, was $52 \pm 5.7\%$ (n = 3)). Therefore, it seems that the peroxide-mediated effects on the EGFR are similar in several ways to the normal functioning of the EGF/EGFR system.

4. Discussion

In this study, we have shown that in response to acute exposure of PC12 cells to hydrogen peroxide, multiple ERK1/2activating mechanisms can be stimulated. We demonstrated that hydrogen peroxide exposure resulted in the elevation of the phosphotyrosine status of the non-receptor tyrosine kinase c-Src as well as the EGFR and Pyk2. Multiple studies have demonstrated that alterations in cellular oxidative state are a potent modulator of multiple diverse and interconnected intermediary cell metabolism signaling mechanisms [13, 30, 31]. Tyrosine kinase signaling systems are typically associated with the creation of multiprotein complexes. These complexes are involved in the control of growth factor or nuclear hormone receptor activity that subsequently stimulates MAPK pathways, which are strongly linked to long-term genotropic hormonal effects, such as cell survival or stress resistance [25, 32-34]. The intersection between tyrosine kinase signaling systems and peroxide-mediated signaling also appears to strongly regulate

nongenotropic actions of mitogen-activated protein kinases (formerly known as microtubule-associated protein kinases), for example, cytoskeletal remodeling and adherence control [23, 35, 36]. One of the important features common to the tyrosine kinase factors we have identified to be activated in our PC12 cells by peroxide, that is, c-Src, EGFR, and Pyk2, is their ability to act as scaffolding proteins for MAPKactivating complexes [25, 37-40]. While all of these proteins are diverse in their nature, for example, EGFR activation requires dimerization and internalization to induce activation of many downstream effectors, Pyk2 activation often appears calcium dependent, and c-Src requires disruption of stable intramolecular interactions to become fully active [25, 41–45], they all demonstrate an important requirement for auto-tyrosine phosphorylation. With regards to this, we demonstrated that in each case, peroxide exposure was able to induce this posttranslational modification (Figures 1(c) and 4(a)). Therefore the action of peroxide in this instance is highly reminiscent, with regards to cell signaling, of the activation of cell surface receptors linked to these signaling pathways. This posit was reinforced by our finding that catalase-mediated degradation inhibited the ability of the exogenous hydrogen peroxide to activate ERK1/2 and tyrosine kinase systems (Figure 2(g)) [46, 47]. All of the peroxide-stimulated signaling factors we identified (c-Src, EGFR, Pyk2) have been strongly implicated as intermediaries in receptor-mediated MAPK/ERK1/2 activation [25, 47-50], again suggesting a reasonable analogy between the effects of exogenous peroxide and receptor-biological-like activity. Another important aspect of receptor-mediated activity is the controllable response to cell stimulation by extracellular ligands. Most receptor-based systems demonstrate rapid tachyphylaxis to the presence of excessive or repeated exposure to stimulating ligands [51, 52]. In recent years, the complexity of these regulatory mechanisms and their ability to modulate the signaling nature of the stimulus have been partially revealed. The receptor desensitization mechanisms are now considered to function as "conditioners" of the stimulating signal, rather than just blunt attenuators of the signal [38, 53-55]. Using our "acute-recovery" peroxide stimulation protocol, we were able to investigate the capacity of PC12 cells to respond to repeated peroxide exposure. When we investigated the ability of peroxide to stimulate ERK1/2 activation, we noted that with four successive peroxide exposures there was a significant reduction in the PC12 responsiveness to peroxide (Figures 3(c) and 3(d)). Interestingly, there seemed to be a specificity of the chemical sensitivity of the extant ERK1/2 response (R4) following the multiple stimuli compared to the initial response in naive cells (R1). Hence, for the R4 response point, the inhibitory effects of AG1478 preincubation upon peroxideinduced ERK1/2 activation were significantly diminished (Figure 4(d)). This reduced EGFR kinase dependence of the peroxide-induced R4 response was correlated to a significant reduction in the degree of peroxide-induced EGFR tyrosine phosphorylation (Figure 4(b)), attenuated peroxide-induced association of EGFR with Grb2 (Figures 5(a) and 5(d), and a strong redistribution of EGFR away from the plasma membrane to the cytosol (Figures 6(e) and 6(i)). In contrast to this, the activation of Pyk2, its physical association with Grb2, and its subcellular localization were not significantly affected from the R1 to the R4 response point. This suggests that while peroxide stimulation can employ diverse tyrosine kinase scaffolds, the relative contribution of these to the signaling effects of peroxide is sensitive to the context of the oxidative stress. While we have focused on a limited number of downstream targets, it is highly likely that other effector outputs of peroxide stimulation could be differentially affected by repetitive exposures in a similar way to more classical "receptorsignaling" systems [38, 56-58]. The mechanisms by which oxidative stress and exposure are linked to ERK1/2 activity are clearly numerous and complex and therefore are probably subject to multiple levels of regulatory control. With respect to this, one primary stress-controlled ERKregulatory mechanism may involve the MAPK phosphatases [MKPs: 59]. MKPs, which belong to the dual-specificity family of protein tyrosine phosphatases (DUSP), negatively regulate the phosphorylation and therefore activity status of kinases such as ERK1/2 [59, 60]. MKP-1, also known as DUSP1, is the prototypic member of the MKP family and has been demonstrated to become activated in response to oxidative stress [61, 62]. Through this regulatory capacity, it is not surprising that MKPs have been associated with cell growth, senescence and aging [63, 64]. Many cells therefore potentially possess a complex network of evolutionarily conserved response modalities linking oxidative stress and MAPK regulation. In future studies, we may yet further appreciate the true signaling depth and complexity of the chemically minimal signaling molecules such as hydrogen peroxide. In addition to this, the functional signaling capacity, which may include control of receptor or nonreceptor transmembrane tyrosine phosphatases [65, 66], of hydrogen peroxide could be targeted to control oxidative stress responses in a similar manner that has been so successful for GPCRs.

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Review Article

Mos in the Oocyte: How to Use MAPK Independently of Growth Factors and Transcription to Control Meiotic Divisions

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In many cell types, the mitogen-activated protein kinase (MAPK) also named extracellular signal-regulated kinase (ERK) is activated in response to a variety of extracellular growth factor-receptor interactions and leads to the transcriptional activation of immediate early genes, hereby influencing a number of tissue-specific biological activities, as cell proliferation, survival and differentiation. In one specific cell type however, the female germ cell, MAPK does not follow this canonical scheme. In oocytes, MAPK is activated independently of growth factors and tyrosine kinase receptors, acts independently of transcriptional regulation, plays a crucial role in controlling meiotic divisions, and is under the control of a peculiar upstream regulator, the kinase Mos. Mos was originally identified as the transforming gene of Moloney murine sarcoma virus and its cellular homologue was the first proto-oncogene to be molecularly cloned. What could be the specific roles of Mos that render it necessary for meiosis? Which unique functions could explain the evolutionary cost to have selected one gene to only serve for few hours in one very specific cell type? This review discusses the original features of MAPK activation by Mos and the roles of this module in oocytes.

1. Introduction

In many cell types, the mitogen-activated protein kinase (MAP kinase/MAPK) also named extracellular signalregulated kinase (ERK) is activated in response to a variety of extracellular growth factor-receptor interactions at the cell surface and leads to the transcriptional activation of immediate early genes. Briefly, tyrosine kinase receptors and p21^{Ras} recruit the Ser/Thr kinase Raf-1 that activates MEK (MAP/ERK kinase), which in turn phosphorylates and activates MAPK, hereby influencing a number of tissuespecific biological activities in diverse cell types, as cell proliferation, survival, and differentiation. In one specific cell type, however, the female germ cell, MAPK does not follow this canonical scheme. In oocytes, MAPK is activated independently of growth factors and tyrosine kinase receptors, acts independently of transcriptional regulation, controls the G2-M period of the cell cycle and not the G1-S transition, and is under the control of a peculiar upstream regulator called Mos.

In the early 1980s, Mos was originally identified as the transforming gene of Moloney murine sarcoma virus (v-mos) causing cellular transformation [1-3]. Its cellular homologue was the first proto-oncogene to be molecularly cloned. When ectopically expressed, the c-mos protooncogene product Mos induces oncogenic transformation of somatic cells [1]. Mos is a Ser/Thr kinase whose transforming activity strictly depends on its kinase activity. Given this activity as an oncoprotein, its expression pattern was surprisingly restricted to germ cells. In frogs, birds, and mammals, very low concentrations of c-mos transcripts were detected in brain and testes, but a high level was observed in ovaries, restricted to oocytes [4-6]. The Mos protein is expressed even in a much more restricted manner than its transcripts, both temporally and spatially at a very specific place: it accumulates during the oocyte meiotic divisions and undergoes selective proteolysis upon fertilization in all eumetazoan except the nematode Caenorhabditis elegans where the gene is absent [4, 7-9]. It has to be noted that in mouse and the jellyfish Clytia hemispherica, Mos is also expressed in

developing spermatids [8-10]. This unique pattern of Mos expression strongly suggested that its function is restricted to meiosis. This hypothesis was confirmed in 1988-1989 by a series of pioneer articles of Noriyuki Sagata, George Vande Woude, and their collaborators who proposed that in the frog oocyte, Mos would be an essential regulator of the universal eukaryotic inducer of M-phase of the cell cycle, MPF (M-phase promoting factor), responsible for reinitiation of meiotic division. MPF is a dimer formed of a catalytic subunit, the Cdk1 kinase (cyclin-dependent kinase 1) and of a regulatory subunit, cyclin B. Mos would serve to activate MPF for entry into the first meiotic division, but also to stabilize MPF during the second meiotic division, inducing the arrest of oocyte that awaits sperm entry [7, 11, 12]. Later, the first function of Mos as an activator of MPF and an initiator of oocyte meiotic entry was not found to be conserved in all animal species whereas its role in postmeiotic arrest turned out to be well conserved: Mos holds in check the unfertilized oocvte arrest. This arrest is characteristic of the entire animal kingdom and is critical for the embryonic development as it allows the mature oocyte to await fertilization, preventing the continuation of cell cycles after meiosis and parthenogenetic development. The target of Mos was discovered few years later. In oocytes, Mos is the upstream activator of MAPK which functions through direct phosphorylation of MEK. Therefore, this original signaling module, Mos/MEK/MAPK, is a critical regulator of some of the most important cell divisions in our life: the meiotic divisions that produce the egg.

At first glance, the physiological meiotic functions of Mos appear to be strikingly different from the oncogenic properties of the kinase in somatic cells. The loss of Mos in mouse leads to parthenogenesis and to the development of ovarian teratomas [13, 14], consistent with the idea that mos could be considered as a meiotic tumour-suppressor gene whereas its activity as an oncoprotein is well established in somatic cells. This opens the question of the apparently conflicting effects of the Mos protein, namely, its ability to induce M-phase entry of oocytes, to arrest mitotic cleavage of Xenopus unfertilized oocyte and to transform mammalian fibroblasts. Another question arises from the observation that the functions of Mos in the female germ cell appear to be largely mediated by MAPK. In the animal oocytes, Mos is a MAPKKK, equivalent to the proto-oncogene Raf-1. Although Raf-1 is expressed in oocytes, Mos is selected to activate the MAPK module during meiotic divisions. What could be the specific roles of Mos that render it necessary for meiosis and that cannot be played by Raf-1? Which unique functions could explain the evolutionary cost to have selected one gene to only serve for few hours in one very specific cell type? This paper discusses the original features of MAPK activation by Mos and the roles of this module in oocytes.

2. Biochemical Properties and Evolution of Mos

In human, mouse, and chicken, the *c-mos* genomic locus contains only a single coding exon corresponding to a poorly conserved *src* homology sequence that yields a protein of

39 kDa. There are two types of conserved regions among Mos proteins: those conserved among all members of the src kinase family that are important for maintaining the basic structure for kinase activity and those conserved only among the Mos proteins [6]. The mutations of Mos that cause the loss of its kinase activity (e.g., substituting an arginine for the conserved lysine residue (K90) found in the ATP-binding site) abolish all its biological functions, meiotic induction, meiotic egg arrest, and transforming activity [15, 16]. Several studies have revealed the contribution of some regions of the sequence to Mos functions, as the helix C laying in the kinase domain whose orientation could govern Mos kinase activity [17, 18]. Mutagenic analysis outside the kinase domain showed that the cytoplasmic localization of the protein is important for its biological activity, as the deletion of a 10 amino acids region required for nuclear localization greatly enhances the transforming activity of Mos [19]. Mos also exhibits an in vitro DNA-binding activity [20] that seems not to be required for any of its biological functions.

2.1. V-Mos versus C-Mos. Mos was originally identified as the transforming gene of Moloney murine sarcoma virus of which several viral isolates have been characterized [1, 3, 21]. Sequence comparison of different v-Mos proteins with murine c-Mos revealed the presence of additional 31 amino acids at the N-terminus of the v-Mos proteins derived from the viral env gene and from an upstream extension of the c-mos open reading frame. Apart from this N-terminal extension, the amino acid sequences of v-Mos and c-Mos either are identical or differ in few amino acids, depending on the viral strains [3, 18]. In vivo, both c-Mos and v-Mos are able to cause oocyte maturation, meiotic oocyte arrest, and transformation of somatic cells [22, 23]. Interestingly, Mos genes are able to transform mouse NIH 3T3 cells with markedly different efficiencies depending on the species but not related to the viral origin of the gene: v-Mos and mouse and chicken c-Mos are equally efficient but more than human and Xenopus c-Mos [16]. In vitro, v-Mos is able to autophosphorylate whereas in parallel experiments c-Mos is not, suggesting a correlation between the transforming activity of v-Mos and its ability to autophosphorylate. However, several point mutations in v-Mos resulted in mutants retaining transforming activity but lacking autophosphorylating activity, showing the functional uncoupling of autophosphorylation and transformation ability [17, 24]. In summary, c-Mos and v-Mos do not exhibit very striking differences in both sequences and cellular activities.

2.2. Mos Is a MAPKKK. Given the critical role played by Mos in animal oocyte meiosis and its oncogenic properties, the identification of its targets was of prime importance. In 1993, several laboratories discovered that in *Xenopus* oocytes, oocyte extracts and mammalian cultured cells, either endogenous or exogenous Mos can activate MAPK by directly phosphorylating and activating MEK1, an immediate upstream activator of MAPK [25–28]. The two amino acids phosphorylated by Mos in MEK are identical to those phosphorylated by Raf-1 [29, 30]. Inside the Mos/MEK/MAPK module, both activation reactions (the phosphorylations of MEK and MAPK) require two phosphorylations on the downstream kinase, producing a cascade in which the activity of MAPK varies as a fourth power of the activity of Mos, so that a modest increase in Mos, as doubling its activity, changes the activity of MAPK from 10% to 90%. The Mos/MAPK cascade is, therefore, ultrasensitive, explaining how the activation of MAPK switches from off to on in response to the minute amount of viral Mos protein present in transformed mouse cells (0.0005% of total protein) or in Xenopus matured oocytes (200 pg or 0.001% of total protein), accounting for the all-or-none character of this cell fate switch [7, 31]. However, during Xenopus oocyte maturation, progesterone induces the synthesis of Mos before MPF activation whereas MAPK, which preexists in immature oocytes is activated at time of MPF activation, that is one or two hours after Mos synthesis has begun [7, 25, 32]. This observation is difficult to reconcile with the all-or-none and ultrasensitive response of MAPK activation to low amounts of Mos. It is possible that initial synthesized Mos is kept at a too low level to activate the cascade, until its stabilization is achieved by MPF and allows the formation of suprathreshold levels required for turning on the MEK/MAPK module [33].

2.3. Other Substrates for Mos? Other proteins have been proposed to be direct targets for Mos since they can be phosphorylated by Mos in vitro. Among them is cyclin B2, the main Cdk1 partner forming the pre-MPF complexes in Xenopus immature oocytes. Cyclin B phosphorylation by Mos has been proposed to be necessary for activation of MPF kinase during Xenopus oocyte maturation and to prevent degradation of cyclin B during the meiotic arrest [34], a model that has never been experimentally proved. Mos has also been described as a tubulin-associated protein kinase [35–37]. Interestingly, in oocytes from mouse, Xenopus, starfish, and the jellyfish Clytia hemispherica, Mos activity is required for the correct formation and peripheral positioning of the meiotic spindle [8, 9, 38, 39]. Mos is also localized on mitotic spindle and spindle pole regions in Mos-transformed NIH/3T3 cells [35, 36]. The tubulin kinase activity of Mos could thus participate in the modification of microtubules and contribute to the formation of the spindle. However, it is not known whether these in vitro substrates do mediate the physiological functions of Mos. It has also been proposed that in mouse oocytes, Mos would contribute to MAPK activation not only through MEK activation but also through the inhibition of an unidentified phosphatase [40], an interesting observation that has not been noted in other species until now.

2.4. Mos Evolution. For more than 20 years, the studies on Mos have been conducted in vertebrates, mainly mouse, human, birds, and frogs. In the entire animal kingdom, to maintain ploidy through successive generations, meiosis must be followed by mitosis after the recovery of diploidy by fertilization. The coordination from meiotic to mitotic cycle is ensured by a meiotic arrest of the oocyte, while the cell awaits fertilization. This arrest occurs at metaphase of the second meiotic division (metaphase II) in vertebrates whereas the stage of oocyte meiotic arrest is variable in invertebrates. It was clearly established in vertebrates that Mos is essential to arrest oocyte meiotic divisions at metaphase II, leading to the hypothesis that Mos is a molecular regulator of MPF and the M-phase of the cell cycle. For this reason, it was assumed that Mos functions only in vertebrate oocytes, until Tachibana and his collaborators isolated the first invertebrate Mos from starfish and demonstrated that it is essential for the natural arrest of the echinoderm unfertilized egg, in G1 phase after completion of meiotic divisions [8]. These results changed the view on the role of Mos. It is not restricted to the maintenance of the metaphase II arrest but more broadly prevents the meiotic/mitotic conversion by arresting the unfertilized oocyte at various stages of meiosis depending on species. The proposal of a conserved role of Mos in invertebrate and vertebrate oocytes was then questioned by the observation that the mos gene is absent in the nematode Caenorhabditis elegans genome and that the Drosophila Mos ortholog is not essential for meiosis [41]. However, in the sawfly, where Mos has also been characterized, it mediates the physiological metaphase I arrest characterizing the insect oocytes [42]. A recent phylogenetic survey reconciled these data by demonstrating that Mos appeared early during animal evolution as an oocyte-expressed kinase and functioned ancestrally in regulating core specializations of female meiosis [9]. Unexpectedly, cnidarian genomes contain more than one mos gene after ancestral duplications, in contrast with bilaterians, in which only one single gene is found. Changes in the characteristics of oogenesis probably explain this diversification of mos genes and their related functions in cnidarians. All genomes from eumetazoan (bilaterian + ctenophore + cnidarian) species examined by Amiel et al. [9] contained mos gene orthologs, showing well-conserved kinase domains, with the exception of C. elegans where the gene was secondarily lost. Mos genes were not detected in available sponge (Amphimedon), choanoflagellate, or fungi genomes, suggesting that the gene may have originated in a common eumetazoan ancestor [9]. Thus, Mos kinases should be seen not as core regulators of meiosis, which is a much older process than Mos origin, but of a particularity of meiosis in eumetazoan. Which innovations of oocyte meiotic divisions relate to mos gene evolution have to be investigated.

3. Oocyte Meiotic Maturation: The Unique Physiological Process under Mos Control

In the animal kingdom, the oocytes growing in the ovaries are arrested at prophase of the first meiotic division that resembles a G2-phase of the cell cycle. These immature oocytes require a physiological stimulus to undergo meiotic maturation: the progression through the meiotic divisions that converts them into fertilizable oocytes, again arrested at various stages of meiosis and awaiting fertilization (Figure 1). Indeed, the embryonic development cannot begin until completion of the female germ cell meiotic divisions. This



FIGURE 1: Progression through meiosis and timing of fertilization: what does Mos do? Oocytes are arrested at prophase of the first meiotic division (prophase I). Under response to a physiological stimulus, MPF is activated and promotes breakdown of the nuclear envelope (GVBD for germinal vesicle breakdown) and formation of the metaphase I spindle. In insects, molluscs, and ascidians, oocytes arrest at metaphase I until fertilization. In the other cases, oocytes extrude the first polar body and enter the second meiotic division. In vertebrates, they arrest at metaphase II until fertilization. In echinoderms and cnidarians, they complete the second meiotic division by emitting the second polar boy, reform a nucleus (female pronucleus), and stop at the G1 phase of the first cell cycle until fertilization. In different species including the nematode *Caenorhabditis elegans*, fertilization occurs at prophase I and corresponds to the stimulus promoting meiotic maturation. Mos has been implicated: (i) in the initial step of MPF activation during reinitiation of meiotic division, (ii) during the metaphase I to metaphase II transition for the suppression of S-phase, for the microtubular spindle organization and for the reactivation of MPF to enter meiosis II, and (iii) in the arrest of oocyte maturation before fertilization.

temporal coupling is ensured by the arrest of meiotic divisions of the oocyte that depends on a biological activity called CSF (for cytostatic factor) [43, 44]. The CSF arrest is released by fertilization. Oocytes arrest at metaphase I in insects, molluscs and ascidians and at metaphase II in vertebrates. In echinoderms and cnidarians, oocvtes complete meiosis and arrest in G1 (and are then called "eggs" in these species, as they completed meiotic divisions). In different species including the nematode *Caenorhabditis* elegans, fertilization occurs at prophase I and corresponds to the stimulus promoting meiotic maturation. Except for this last case, the stimuli for maturation are provided at ovulation by the follicular cells surrounding the oocyte. The signals are very different from species to species: steroid hormones in frogs and fishes, modified purines in starfish, removal of a follicular inhibitor in mammals, but all activate signaling pathways that converge to the same target, independently of transcription: the activation of the universal eukaryotic inducer of M-phase, MPF, a complex formed of the Cdk1 kinase, and Cyclin B. Once activated, MPF promotes entry into the first meiotic division: breakdown of the nuclear envelope (known as GVBD for germinal vesicle breakdown) and formation of the metaphase I spindle (Figures 1 and 2). MPF activity falls during anaphase I, due to partial cyclin B degradation, and rises again leading to entry into meiosis II. Importantly for the generation of proper haploid gametes, DNA replication does not occur between both meiotic divisions. The need for the Mos/MAPK cascade during oocyte meiotic maturation has been debated for decades (Figure 1). First, Mos has often been implicated

in the initial step of MPF activation during reinitiation of meiotic division, especially in the frog oocyte. Second, Mos has been shown to be required during the metaphase I to metaphase II transition for the suppression of S-phase and for the reactivation of MPF after meiosis I, thus enabling the oocyte to enter meiosis II. Third, a universal role of Mos is to prevent parthenogenesis by arresting oocyte maturation at the various stages depicted in Figure 1, allowing them to await fertilization. Mos is therefore a key regulator of meiosis in the animal kingdom.

4. Regulation of Mos Activity

Mos protein functions as a kinase in a meiosis-specific manner in animal oocytes. In *Xenopus*, *Mos* gene is actively transcribed in the grownup oocytes where its message is abundant [6]. However, oocytes arrested at prophase I lack detectable levels of Mos. The synthesis of the protein is induced in response to the physiological stimulus that promotes reentry into meiotic divisions, Mos protein then accumulates throughout meiotic maturation, is stably maintained in the mature oocyte, and is finally degraded at fertilization (Figure 2). This unique pattern of Mos expression, accounting for its restricted function during oocyte meiosis, is clearly under a tight translational and proteolysis control.

4.1. Translational Control of Mos. Many studies have been devoted to the regulation of translation of maternally stored

mRNAs during meiosis resumption of *Xenopus* oocytes. In prophase-arrested oocytes, translation is repressed. Progesterone, the physiological inducer of meiotic divisions in *Xenopus*, induces the ordered translation of mRNAs based on polyadenylation events. This translational regulation depends on regulatory elements within the 5' and 3' untranslated regions (UTRs) of target mRNAs which are recognized by sequence-specific RNA protein complexes to mediate translational control [45, 46].

In immature Xenopus oocytes, the kinase TOR (target of rapamycin) controls the translation of RNAs through a 5'TOP (terminal oligopyrimidine) sequence that contributes to suppress translation of other RNAs, including Mos mRNA, until hormonal stimulation of maturation [47]. The translational induction of the dormant mRNA encoding Mos occurs 2 to 3 hours after stimulation by progesterone, before MPF activation [7, 12] (Figure 2). The 3' end polyadenylation of Mos mRNA and a 5'end modification, cap-specific 2'-Omethylation, were shown to be pivotal regulatory steps for translational recruitment and for the progression of Xenopus oocytes through meiosis [48, 49]. Cytoplasmic polyadenylation requires two sequences in the 3'UTR of Mos mRNA, the U-rich cytoplasmic polyadenylation element (CPE) and the near-ubiquitous polyadenylation hexanucleotide AAUAAA recognized by the multifactor complex CPSF (cleavage and polyadenylation specificity factor). CPE is recognized by a group of factors among them the two most important are CPEB, the CPE-binding factor, and Maskin [50-52]. In oocytes, Maskin also binds eukaryotic translation initiation factor 4E (eIF4E), an interaction that excludes eIF4G and prevents formation of the eIF4F initiation complex [51]. It has been proposed in *Xenopus* that an early site-specific phosphorylation of CPEB, possibly catalyzed by the Aurora-A kinase [53], would be essential for the polyadenylation of Mos mRNA by the poly(A)polymerase. Once cytoplasmic polyadenylation has been promoted by CPEB, the newly elongated poly(A) tail becomes bound by poly(A)-binding protein (PABP), which in turn binds eIF4G and helps to displace Maskin from eIF4E, thereby inducing translation [51, 52]. Polyadenylation requires two factors, symplekin, a CPEB- and CPSF-binding protein that serves as a scaffold upon which regulatory factors are assembled, and xGLD-2, an unusual poly(A) polymerase that is anchored to CPEB and CPSF even before polyadenylation begins [54].

However, several findings refute the hypothesis that CPE sequences and CPEB alone could account for the range of temporal inductions of maternal mRNAs, including Mos mRNA, observed during *Xenopus* oocyte maturation [55]. Polyadenylation and mRNA translational activation of Mos are also controlled by a distinct CPE-independent mechanism that depends on a 3'UTR polyadenylation response element (PRE) [55, 56]. This translational activation is mediated by the transacting factor Musashi that binds to the PRE of *mos* mRNA [56, 57]. As CPEB, Musashi would also be essential for Mos translational activation during *Xenopus* oocyte meiotic maturation [58]. Several reports suggest that mRNA translation directed by CPE is a late event that would require early Musashi-dependent mRNA translation, implying that Musashi function is necessary to establish

the temporal order of maternal mRNA translation during meiotic progression [57–59]. It is, therefore, still difficult to get a clear picture of the signaling events that trigger Mos mRNA polyadenylation and translation in the *Xenopus* oocyte.

Interestingly, MAPK can stimulate Mos synthesis in *Xenopus* oocyte, creating a positive feedback loop. Microinjection of activated forms of MEK or MAPK is sufficient to stimulate Mos mRNA polyadenylation and translation [60, 61] whereas inhibition of MAPK activation prevents Mos accumulation [62]. MAPK activity could contribute to CPEB phosphorylation and activation [63], but the precise mechanism allowing this kinase to stimulate the polyadenylation of Mos mRNA is still elusive.

In starfish, rat, and mouse, the synthesis of Mos is also initiated in maturing oocytes, except that it accumulates only after MPF activation and the first meiotic reentry [8, 64, 65] (Figure 2). This noticeable difference in the translational timing of Mos mRNA in mouse and starfish oocytes compared to Xenopus oocyte, explains why Mos is not involved in the activation of MPF and the entry into the first meiotic division in these species [8, 13, 14, 38], a notso-surprising result given that this process does not depend on protein synthesis. Similarly to the translational regulation described in the Xenopus oocyte, mouse Mos mRNA is under the translational control of cytoplasmic polyadenylation, a necessary event for the oocyte progression to meiosis II after the first polar body emission. Cytoplasmic polyadenylation of Mos mRNA in murine oocyte requires three cis elements in the 3' UTR, the polyadenylation hexanucleotide AAUAAA, and two CPEs [66]. The biochemical events that govern polyadenylation in mouse oocytes are not well known, but they also involve CPEB whose activity is controlled by Aurora-catalyzed phosphorylation, similarly to the Xenopus situation [67–69].

Interestingly, one of the two Mos paralogs of the jellyfish *Clytia hemispherica* is subject to differential translational regulation, being expressed during the growth period of oogenesis, before meiotic maturation, perhaps under the control of the TOR pathway [9]. This raises the attractive possibility that Mos may have acquired new roles during evolution after sequence changes in the UTRs affecting translational timing [70, 71]. Acquisition of a 5'TOP sequence in one Clytia paralog may have resulted in an earlier translational timing of this paralog compared to the other one, leading to functions during the oocyte growth period preceding meiotic maturation. In Xenopus, 3'UTR changes, such as an early acting Musashi PRE, could have resulted in the temporal advancement of Mos translation before MPF activation, explaining its atypical participation in MPF activation and initiation of oocyte maturation. In contrast, the translational activation of the human Mos 3' UTR is uniquely dependent on a late acting CPEdependent process [59]. Mos 3'UTR regulatory differences, therefore, underlie species-specific temporal patterns of Mos mRNA translational recruitment during oocyte maturation and hence different temporal windows for its functions, offering it the possibility to regulate MPF activation, or not.



FIGURE 2: Patterns of Mos expression and MPF activity during oocyte meiotic maturation and at fertilization in *Xenopus*, mouse, and starfish. Prophase I-arrested oocytes require a physiological stimulus to undergo meiotic maturation: progesterone in frogs, release from the follicle in mammals, and 1-methyl-adenine in starfish. Once activated, MPF promotes entry into the first meiotic division: breakdown of the nuclear envelope (GVBD for germinal vesicle breakdown) and formation of the metaphase I spindle (MI). MPF activity falls due to partial cyclin degradation at meiosis I/meiosis II transition or interkinesis (IK), during which chromosomes remain condensed without nuclear membranes and in the absence of DNA replication. MPF rises again leading to entry into meiosis II. In vertebrates, oocytes arrest at metaphase II (MII), while in echinoderms, oocytes complete the second meiotic division and arrest at the G1 phase. Mos translational timing is different among species, occurring before MPF activation in *Xenopus* (however, Mos protein is unstable until GVBD and MAPK activity is detected only at time of MPF activation, not illustrated) and during metaphase I in other species.

4.2. Control of Mos Stability. Even though the synthesis of Mos begins soon after progesterone stimulation in Xenopus oocytes, the protein remains unstable and unable to activate MAPK until MPF activation [33, 72, 73]. The polyadenylation-controlled translation of Mos is an early event but is not sufficient for Mos to accumulate. Several studies attempted to elucidate the molecular mechanisms that govern the metabolic stability of Mos during meiotic maturation. Using a number of Mos mutants expressed in Xenopus oocytes, Nishizawa et al. [33, 74] demonstrated that the instability of Mos depends on the ubiquitination of Lys34 and is determined primarily by its penultimate Nterminal residue, a proline, and the phosphorylation status of the adjacent serine (Ser3) residue. Clearly, Mos is stabilized by phosphorylation at Ser3, its major phosphorylation site in vivo [17]. This critical phosphorylation for Mos stability is catalyzed by MPF kinase activity, which probably acts to prevent the N-terminus of Mos from being recognized by its ubiquitin ligase [33, 74, 75]. Similarly in mouse, the phosphorylation of Ser16 prevents Mos degradation and stabilizes the protein [76]. The unique role of Mos phosphorylation would be to stabilize the protein. Therefore, while the Mos/MAPK pathway can facilitate the activation of MPF, MPF is required to directly phosphorylate and stabilize Mos protein, a mechanism creating a positive feedback loop between Mos and MPF. Importantly, these results reconcile some apparent conflicting conclusions on Mos functions in Xenopus and mouse. In both cases, Mos activity can take place only after MPF activation and meiosis reentry: the protein needs to be stabilized by MPF despite an early translational initiation in the frog oocyte, while it is only synthesized after MPF activation in the mouse oocyte.

4.3. Control of Mos Degradation at Fertilization. In all species studied until now, fertilization induces a rapid escalation in intracellular calcium ion concentration that releases the meiotic arrest. Interestingly, Mos was proposed to be the cytostatic factor responsible for meiotic arrest of the unfertilized oocytes [12] and was shown to undergo specific proteolysis upon fertilization when the arrest is relieved [7, 77]. However, in both Xenopus and mouse oocytes, Mos disappearance at fertilization starts 30 to 45 minutes after the calcium surge [7, 77, 78], whereas MPF inactivation occurs within 15 minutes through cyclin B degradation (Figure 2). Clearly, the degradation of Mos at fertilization is not required for cyclin B proteolysis and is, therefore, not the inducer of the release of the meiotic arrest but rather a consequence of this exit [79]. To summarize, Mos is essential for establishing and maintaining the meiotic arrest; the release of this arrest at fertilization is due to a mechanism that overcomes Mos but is not a consequence of Mos degradation. In Xenopus oocytes, MPF ensures Mos stability by phosphorylating its Ser3 residue. At fertilization, cyclin B is rapidly degraded, leading to the inactivation of MPF and consequently the dephosphorylation of Mos. Under this unphosphorylated state, Mos is then degraded by a yet unidentified ubiquitin ligase, different from the anaphase-promoting complexcyclosome (APC/C) that targets several cell-cycle regulatory proteins, including cyclin B, for destruction [33, 74, 75]. However, the destruction of Mos under the control of MPF appears to be specific to vertebrate oocytes that are arrested in M-phase. It would be important to evaluate how unfertilized eggs of invertebrates, arrested after meiosis completion in G1, get rid of Mos, an essential event to prevent the organism from the oncogenic activity of this protein.

5. Which Functions for Mos in Oocytes?

5.1. Mos as an Initiator for Oocyte Maturation? The very first studies on the physiological function of Mos were conducted on the Xenopus oocytes and revealed that injection of Mos antisense oligonucleotides blocks GVBD and MPF activation whereas the injection of Mos RNA activated MPF and induced GVBD in the absence of progesterone [6, 11]. In contrast to mouse or starfish, a period of protein synthesis is necessary for MPF activation in the frog oocyte [80]. Yew et al. [81] reported that Mos protein efficiently induces GVBD and the activation of MPF in the absence of protein synthesis (but in the presence of low concentrations of progesterone unable to trigger meiotic maturation), leading to the conclusion that Mos is the only synthesized protein required for initiating maturation. All constitutively active downstream effectors of Mos, MEK, MAPK, and p90^{Rsk}, are also able to induce meiotic maturation when microinjected into oocytes [82-84]. The effects of Mos on MPF activation are mediated through MEK/MAPK/p90^{Rsk}, as microinjected Mos is inactive in the presence of the pharmacological MEK inhibitor, U0126 [85]. Altogether, these results led to a simple scenario, where MPF activation is the result of a linear chain of molecular events initiated by progesterone, starting with the synthesis of Mos protein, the subsequent activation of the MEK/MAPK/p90^{Rsk} cascade that would eventually control the two regulators of the Cdk1 catalytic subunit of MPF, the Myt1 kinase that phosphorylates and inactivates Cdk1 and the Cdc25 phosphatase that specifically activates Cdk1 [86-88].

However, this simple view was then questioned by several studies. Gross et al. and Fisher et al. showed that progesterone is able to activate MPF by a mechanism independent of MAPK [85, 89]. This conclusion is difficult to reconcile with a requirement for Mos downstream of progesterone in Xenopus oocyte. Consistent with the idea that MAPK activation downstream of Mos synthesis is not required for maturation, inhibition of Mos synthesis by morpholino antisense oligonucleotides fails to block progesterone-stimulated GVBD [90]. This conflicting results on the requirement of the Mos/MAPK cascade to activate MPF in Xenopus oocyte were recently reconciled. It was shown that MPF activation induced by progesterone is completely abolished when cyclin B synthesis and the Mos/MAPK pathway are simultaneously impaired [88]. The replenishment of at least one of these pathways restores MPF activation. Altogether, these results demonstrate that MPF activation requires either the Mos/MAPK pathway or cyclin B synthesis. Each of these pathways can bypass the deficiency of the other one. In contrast to cyclin B1 accumulation induced by progesterone independently of MPF activation, the strong accumulation of Mos requires a stabilizing phosphorylation catalyzed by MPF [73, 75] and as a consequence MAPK activation only takes place when MPF activation is already initiated. This differential regulation in the accumulation of Mos and cyclin B1 suggests that the physiological pathway induced by progesterone depends on cyclin B synthesis and that the Mos/MAPK cascade contributes to MPF activation once Mos stabilization is achieved. When cyclin B synthesis is impaired, some rescue mechanism could recruit the Mos/MAPK pathway, allowing it to complement the deficiency in cyclin B synthesis. These findings solve the paradoxical situation of the frog oocyte meiotic reentry control, where the Mos/MAPK activation was considered as necessary for MPF activation, while this is clearly not the case in all other systems studied until now (mouse, starfish, jellyfish, *Drosophila*, and *C. elegans*, where Mos is not expressed yet at the time of MPF activation, Figure 2) and integrates the *Xenopus* in the context of a universal mechanism for meiotic maturation in oocytes throughout the animal kingdom.

5.2. Suppression of DNA Replication and Entry into Meiosis II. In Xenopus, the ability to replicate DNA is acquired during maturation at the beginning of meiosis I by synthesis of the only missing replication factor, Cdc6, which is essential for recruiting the minichromosome maintenance (MCM) helicase to the prereplication complex [91, 92]. After GVBD, the maturing oocyte is thus fully equipped with a functional replication machinery that has to be inhibited to prevent the entry into S-phase until fertilization. In Xenopus, Mos has been shown to be required during the metaphase I to metaphase II transition to suppress S-phase. When the synthesis or the activity of Mos is specifically inhibited or when MAPK activation is prevented by U0126, Xenopus oocytes complete meiosis I but a nuclear envelope reforms and DNA replication occurs [85, 90, 93]. Essentially similar results have been obtained in starfish oocytes [8]. However, in mouse oocytes, conflicting results have been obtained concerning the involvement of Mos and MAPK in S-phase suppression and entry into meiosis II. The ablation of Mos by antisense oligonucleotides either arrests oocytes before extrusion of the first polar body [4] or induces nuclear reformation and DNA replication after meiosis I [94, 95], as in Xenopus. In contrast, oocytes from the mos gene knockout mice enter meiosis II essentially normally, despite going through an interphase-like microtubular stage [13, 14, 38]. The reasons for these conflicts could be due to differences in the strains of mice used or to the experimental strategy used for deletion. In jellyfish oocytes where Mos is ablated by morpholino antisense or MAPK activation is inhibited by U0126, GVBD occurs on time, but oocytes show a complete failure to emit both first and second polar bodies. However, they do not reform a replicating nucleus [9]. Then, the ability of Mos to suppress DNA replication between the two meiotic divisions of the oocyte is not a function universally conserved. These differences could be related to the presence or the absence of a functional replicative machinery in oocytes, depending on the species. For instance, mouse oocytes do not acquire the ability to replicate DNA before metaphase II (and therefore do not need to suppress it), in contrast to starfish and Xenopus oocytes that develop the ability to replicate DNA early after GVBD [8, 91–93].

Until now, the molecular mechanism controlled by the Mos/MAPK cascade and leading to the inhibition of DNA replication remains unclear. All the findings support the view of a critical function of Mos at meiosis I-meiosis II transition in vertebrates: the Mos/MAPK module is involved in MPF reactivation that depends both on the arrest of cyclin B degradation, initiated at the exit of meiosis I, and on new cyclin B synthesis, allowing MPF reactivation and entry into meiosis II [85, 90, 93, 96]. By controlling this cyclin B turn-over, Mos allows MPF activation and entry into meiosis II. The Mos/MAPK module could act indirectly on the replication machinery through the control of MPF activity. Since quite similar failure of the meiosis I/meiosis II transition occurred in Xenopus oocytes that were injected either by antisense against Mos mRNA or dominant-negative Cdk1 kinase [93], it was suggested that MPF reactivation occurring under the control of Mos/MAPK after meiosis I would suppress DNA replication. However, when the reactivation of MPF at meiosis II is inhibited specifically by antisense oligonucleotides against B-cyclins, the Xenopus oocytes degenerate, fail to form a second meiotic spindle, but do not support nuclear organization and DNA replication [96]. This careful analysis favors the view that the Mos/MAPK pathway acts directly to suppress DNA replication, independently of MPF activity.

A conserved function for Mos revealed by observations in the oocytes of starfish, mouse, Xenopus, and the jellyfish Clytia is its involvement in the control of spindle formation and positioning and the chromatin organization. This was first revealed by analysis of mouse oocytes [38, 97, 98]. Remarkably in $mos^{-/-}$ oocytes or in oocytes where MEK is inhibited, the microtubules and chromosomes evolve towards an interphase-like state during the transition between two meiotic M-phases and then exhibit monopolar half-spindles [38, 97-99]. Later on, similar observations were performed in other systems [8, 9, 39]. This ancient and conserved role of the Mos/MAPK in the modulation of microtubular cytoskeleton to assure meiotic spindle formation and positioning could contribute to its cytostatic activity independently on the control of MPF in oocytes arrested at metaphase I (as Drosophila) or metaphase II (as in mouse, see next paragraph). It could also contribute to the chromosome instability of tumor cells where mos is upregulated [100].

5.3. The Meiotic Arrest of the Unfertilized Oocyte: A Conserved Function of Mos. In all animals, oocytes halt meiosis to prevent embryonic development in the absence of fertilization. The stage of meiosis at which the oocyte (also called "egg" in species where meiotic divisions are completed, as echinoderms and cnidarians) typically arrests varies depending species: metaphase II in vertebrates, metaphase I in insects, molluscs, and ascidians, G1 phase following meiosis in echinoderms and cnidarians, prophase I in nematodes (Figure 1). Surprisingly, given the diversity of stages where the cell cycle is halted, Mos was found to be the ubiquitous cytostatic factor responsible for the unfertilized oocyte arrest throughout the animal kingdom.

5.3.1. Metaphase II Arrest in Vertebrates. The activity responsible for this arrest was first described in 1971, by injecting extracts derived from metaphase II-arrested oocytes into one blastomere of a 2 cell-stage Xenopus embryo [12, 43]. The injected blastomere arrests at the next mitosis with a metaphase spindle and a high MPF activity. On the basis of these observations, Masui and Markert established the existence of an activity called CSF (cytostatic factor) that is responsible for metaphase II-arrest. Using this assay, Sagata et al. [12] proposed that Mos is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. Since then, the downstream targets of Mos, MEK, MAPK, and p90^{Rsk} were also shown to cause an M-phase arrest when injected in a Xenopus dividing blastomere [101-104]. The ability of Mos to confer a CSF arrest is abrogated when MAPK activity is prevented [101, 104, 105]. Conversely, any upstream activator of MAPK is able to induce a CSF arrest, as the small G protein Ras or the kinase Raf [106, 107]. These results suggest that the metaphase II-arresting CSF activity of Mos is largely mediated by MAPK. Nevertheless, the physiological validation of the Mos/MAPK pathway as CSF came from the deletion of Mos in oocytes. Ablating Mos synthesis by an antisense strategy in Xenopus oocytes induces cell cycle oscillations after meiosis I, mimicking the early embryonic cell cycles [90]. Invalidating the mos encoding gene in mouse leads to a failure of mature oocytes to arrest during meiosis and as a consequence to parthenogenetic activation of unfertilized oocytes [13, 14, 38].

In vertebrates, the Mos/MAPK pathway serves to stabilize MPF, ensuring an arrest at the metaphase stage. Releasing this blockage requires the activity of the APC/C protein complex, a ubiquitin ligase that targets cyclin B for destruction [108, 109]. In mouse and Xenopus metaphase II-arrested oocytes, APC/C is directly inhibited by the Erp1/Emi2 protein [110]. During oocyte maturation, Erp1/Emi2 appears only after metaphase I, explaining why the oocyte does not stop at metaphase I, but at metaphase II [111, 112]. Upon fertilization, a transient rise in free intracellular calcium activates calmodulin-dependent protein kinase II that phosphorylates Erp1/Emi2, thereby creating a docking site for the Polo kinase. The Erp1/Emi2 phosphorylation by Polo kinase targets it to destruction, releasing APC/C from inhibition [110, 113, 114]. As a consequence, cyclin B is degraded, MPF activity is thus inactivated and the fertilized oocyte exits metaphase II. Given that Erp1/Emi2 can itself inhibit APC/C and stabilize MPF, why is the Mos/MEK/MAPK/p90^{Rsk} required for metaphase II arrest? Recent works provided promising clues about the relationships between Mos and Erp1/Emi2. In Xenopus metaphase II-arrested oocytes, Erp1/Emi2 is a substrate of p90^{Rsk}, and Mos-dependent phosphorylation of Erp1/Emi2 by p90^{Rsk} is crucial for both stabilizing Erp1/Emi2 and establishing CSF arrest in meiosis II oocytes [115, 116]. More precisely, the Rsk-mediated phosphorylation of Erp1/Emi2 promotes its interaction with the protein phosphatase PP2A. PP2A dephosphorylates two distinct clusters of residues in Erp1/Emi2, one responsible for modulating its stability during the metaphase II-arrest and one controlling its binding to the APC/C [112, 117, 118]. Thus, Mos and Erp1/Emi2 collaboratively establish and maintain metaphase II arrest in *Xenopus* oocytes (Figure 3).

In mouse, the APC/C Erp1/Emi2 also plays a critical role for maintaining the metaphase II-arrest of the unfertilized



FIGURE 3: Meiotic arrest of the unfertilized oocyte: the downstream effectors of Mos/MAPK. In all species, oocytes halt meiosis to prevent embryonic development in the absence of fertilization. Depending on species, meiosis arrests at prophase I, metaphase I, metaphase II, or G1 following meiosis. Except in *C. elegans*, Mos was found to be the ubiquitous cytostatic factor responsible for the unfertilized oocyte arrest. Its downstream targets accounting for the meiotic arrest of the unfertilized oocytes are indicated.

oocyte [119, 120]. Therefore, it would be logical to predict that the mechanism of metaphase II-arrest establishment is also conserved between frog and mouse. However, this is not so. Surprisingly, although it is well established that the Mos and MAPK are essential for establishing this arrest, p90^{Rsk}, which is activated by MAPK as in the other species [121], is not involved in the metaphase II-arrest of mouse oocytes [122]. This is in strong contrast with *Xenopus* or starfish unfertilized oocytes, where it is the main mediator of Mos cytostatic activity (see before and below). Then, the downstream effector of MAPK regulating APC/C through Erp1/Emi2 and/or stabilizing the microtubular spindle still requires to be elucidated in mouse.

5.3.2. G1 Arrest in Starfish and Jellyfish. In contrast to vertebrates whose oocytes arrest at metaphase II to await fertilization, unfertilized eggs of starfish are blocked at G1 phase after completion of meiosis II. In the absence of Mos, meiosis I is directly followed by repeated embryonic mitotic cycles in starfish [8]. Therefore, despite the difference in the arrest stage between vertebrates and starfish, both arrests depend on the same Mos-MAPK pathway, indicating that the difference relies on particular downstream effectors. Inhibition of p90^{Rsk} activity in the starfish unfertilized egg leads to S-phase in the absence of fertilization [123], indicating that the effects of Mos on the meiosis arrest are mediated, at least partially, through its downstream effector, p90^{Rsk}, as in Xenopus oocyte. In starfish G1-arrested eggs, chromatin is loaded with the MCM complex to form the prereplicative complex. p90^{Rsk} blocks S-phase entry by preventing the further loading of Cdc45 onto chromatin to form the preinitiation complex and the subsequent initiation of DNA replication [124] (Figure 3). However, the S-phase induced by p90^{Rsk} inhibition is not followed by M-phase when MAPK remains active, owing to repression of cyclin A and B synthesis (Figure 3). By contrast, inactivation of MAPK alone induces M-phase. Thus, there is a divergence of separate pathways downstream of MAPK that together block the initiation of the embryonic mitotic cycle. One depends on p90^{Rsk} and prevents S-phase, the other is not mediated by p90^{Rsk} and leads to the prevention of the first mitotic M-phase through suppression of mitotic cyclin synthesis (Figure 3). Release from this dual lock by fertilization results in the start of the embryonic cell cycle [125].

In the jellyfish *Clytia hemispherica* as in starfish, unfertilized eggs are blocked in G1 phase after meiotic division completion and the invalidation of the Mos/MAPK pathway leads to parthenogenetic development with complete cleavages, revealing that the Mos/MAPK function in meiotic arrest is conserved through the animal kingdom [9].

5.3.3. Metaphase I Arrest. In many invertebrates, as insects, molluscs, and ascidians, oocytes arrest at metaphase I. At first glance, it is tempting to speculate that the molecular basis of this arrest could resemble those operating during the metaphase II-arrest in vertebrates oocytes, based on the Mos/MAPK pathway as an upstream regulator of cyclin B stabilization through APC/C inhibition. This is not so. The Drosophila homolog of Mos has been identified and is expressed specifically in oocytes. As in vertebrates, Mos is responsible for the majority of MAPK activation that occurs during meiotic maturation. Unexpectedly, the oocytes with a Mos deletion complete meiosis normally and produce fertilized embryos that develop [41]. Therefore, the Drosophila Mos ortholog would not be essential for meiosis. Some innovation in oocyte function could explain the relaxation of the cytostatic role of Mos in Drosophila, as the adoption of internal fertilization that could reduce the delay between meiotic completion and fertilization. It has also to be noticed that the mechanism by which the metaphase I arrest is maintained and released is particularly intriguing in Drosophila. It has been demonstrated that chiasmata are essential for signaling the arrest [126, 127], but the role of MPF remains unknown. Then, the molecular mechanisms underlying the metaphase I arrest could regulate very different targets from those identified in other species. In the parthenogenetic insect *Athalia rosae*, the Mos/MAPK module arrests the unfertilized oocytes at metaphase I [128]. Therefore, the role of Mos in *Drosophila* oocyte cannot be extrapolated to all insects (Figure 3).

The oocytes of the ascidian *Ciona intestinalis* merge as a new model to study the meiotic divisions. The Mos/MAP kinase pathway is perfectly conserved in ascidians and metaphase I-arrested oocytes of *C. intestinalis* contain a CSFlike activity able to block cell division in two-cell embryos [129]. MAPK might ensure this activity [130], suggesting that meiotic arrest in *C. intestinalis* could resemble that of vertebrates, such as *Xenopus*, accordingly to the position of this organism in the evolutionary tree (Figure 3).

5.3.4. C. elegans: Meiotic Arrest without Mos. The Caenorhabditis elegans nematode has secondarily lost the mos gene. In this worm, MAPK is activated at two separate steps during the oocyte meiotic cell cycle, under the control of the small G protein Ras and the kinase Raf that replaces Mos [131, 132]. It is first stimulated in the pachytene stage of meiotic prophase I, where it is required for the progression through pachytene. MAPK is then inactivated rapidly after pachytene and remains inactive throughout diakinesis, which is the point of prophase arrest in C. elegans oocyte. The prophase arrest is relieved by a maturation signal produced by the sperm that leads to MAPK reactivation [133]. Interestingly, maintaining MAPK in an inactive state after pachytene exit is necessary to allow the developing oocytes to arrest the cell cycle in diakinesis until maturation is induced by the sperm signal. Oocytes with a constitutive MAPK activity after pachytene completion are unable to arrest in diakinesis for a prolonged time, and they enter a mitotic cell cycle without being fertilized [134]. Therefore, despite the lack of mos gene in C. elegans, MAPK exerts a function during meiosis, being required in oocytes to coordinate meiosis progression with fertilization. However, instead of playing the usual cytostatic role, active MAPK is necessary to break the meiotic arrest where the C. elegans oocyte is awaiting fertilization (Figure 3). This observation is not so surprising given the fact that the arrest where the C. elegans oocyte awaits fertilization corresponds to the universal arrest, where oocytes await the meiotic maturation signal, prophase I, characterized by the absence of Mos expression through all the animal kingdom.

6. Concluding Remarks

Since the discovery of the meiotic functions of Mos about 20 years ago, there has been many studies exemplifying the apparently conflicting effects of the Mos protein, namely, its ability to induce M-phase entry of oocytes, its ability to arrest the meiotic cell cycle at various stages, and its ability to transform mammalian fibroblasts. As reviewed here, these studies can be easily reconciled when taking into account three characteristics of Mos that distinguish it from the main somatic regulators of MAPK, as Raf-1.

First, Mos activates MAPK independently of the presence of serum, growth factors, and tyrosine kinase receptor activation. In this regard, it acts like a dominantly acting oncogene.

Second, its kinase activity is not regulated by posttranslational modifications. Once the protein is expressed, it is active. The regulation of its expression, depending on translation and degradation, determines where and when it will function.

Third, Mos appeared early during animal evolution as a specific oocyte-expressed kinase. A particularity of full-grown oocytes is that transcription of genes is silent: transcriptional activities stop at the end of the growth period and are reinitiated in the cleaving embryos, after fertilization. In this oocyte physiological background, even if the Mos-MAPK pathway would phosphorylate and activate transcription factors, this would not induce transcription of any genes in the oocyte.

From these observations, the apparent conflicting nature of Mos, acting physiologically as a tumour-supressor gene in the unfertilized oocyte, and as an oncogene when inappropriately expressed in somatic cells, seems to be basically resolved: the functions played by Mos depend on the identity of the final MAPK targets that are at its disposal.

When expressed in somatic cells, MAPK activated by Mos can phosphorylate and stabilize transcription factors as c-Fos and c-Myc, leading to transcriptional induction of critical oncogenes and cellular transformation [135, 136]. Clearly, this cannot happen in oocyte where transcription is inactive. It is also probable that Mos can impose a meioticlike phenotype on all stages of the somatic cell cycle. In particular, its meiotic regulatory activities concerning the formation of microtubular spindles, the cohesion of sister chromatids and the omission of S-phase, could participate to the chromosome instability characterizing malignant clones where meiotic genes as *mos* are induced [100].

Physiologically, Mos functions in regulating core specializations of female meiosis. Asymmetric spindle positioning and polar body emission as well as cytostatic arrest are the ancestral functions of Mos for Eumetazoa. Interestingly, despite the differences in the meiotic arrest stages of unfertilized oocytes among species, all of them are under the control of Mos, indicating again that the differences rely on particular downstream targets of MAPK. If the translational regulation of Mos is modified, in time or in space, Mos can acquire new roles by finding new targets. This scenario could have operated in the oocyte during evolution, for example, after sequence changes in its UTRs affecting translation timing.

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Review Article

The Role of Specific Mitogen-Activated Protein Kinase Signaling Cascades in the Regulation of Steroidogenesis

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Mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine kinases that are activated by a large variety of extracellular stimuli and play integral roles in controlling many cellular processes, from the cell surface to the nucleus. The MAPK family includes four distinct MAPK cascades, that is, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, c-Jun N-terminal kinase or stress-activated protein kinase, and ERK5. These MAPKs are essentially operated through three-tiered consecutive phosphorylation events catalyzed by a MAPK kinase kinase, a MAPK kinase, and a MAPK. MAPKs lie in protein kinase cascades. The MAPK signaling pathways have been demonstrated to be associated with events regulating the expression of the steroidogenic acute regulatory protein (StAR) and steroidogenesis in steroidogenic tissues. However, it has become clear that the regulation of MAPK-dependent StAR expression and steroid synthesis is a complex process and is context dependent. This paper summarizes the current level of understanding concerning the roles of the MAPK signaling cascades in the regulation of StAR expression and steroidogenic cell models.

1. Introduction

For the control of diverse signaling in response to the extracellular milieu, cells develop sophisticated tools to transmit the appropriate signals and thereby orchestrate the responses. The signaling mechanism of an agent involves effector-receptor coupling, production of second messengers, activation of protein kinases, and distribution of these transducers to specific intracellular targets. Mitogenactivated protein kinases (MAPKs) are the serine/threonine kinase family of conserved enzymes that are considered to be the central building blocks in the intracellular signaling networks [1-5]. There are more than a dozen of MAPKs known in mammals, and these enzymes exist in several isoforms (Table 1). Mammals express four distinctly regulated groups of MAPKs, that is, extracellular signalregulated kinase 1/2 (ERK1/2), p38, c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), and ERK5/Big MAPK (BMK1) [2, 3, 6]. Each of these four MAPK cascades consist of at least three tiers of protein kinases that are consecutively activated by phosphorylation events:

MAPK kinase kinase (MAPKKK or MAP3K or MEKK) activates MAPK kinase (MAPKK (MKK or MEK) or MAP2K), which in turn activates MAPK. The latter phosphorylates a large array of substrates, including MAPK-activating protein kinases (MAPKAPKs) [7-9]. Even so, the different tiers are composed of many similar isoforms that can be activated by more than one MAPK, increasing the complexity and diversity of MAPK signaling. Substrates, regulation, and function of different MAPKs have been discussed in many review articles [1, 2, 4, 5, 9–11], and will not be elaborated upon in great detail here. MAPKs are important signal transducing enzymes that are involved in transmitting signals from a wide variety of extracellular stimuli including those of growth factors, hormones, cytokines, and neurotransmitters. In fact, MAPKs are major components of signaling pathways regulating a large array of intracellular events, such as proliferation, differentiation, development, acute signaling in response to hormones, stress response, programmed cell death, gene expression, and steroidogenesis [1, 2, 12-14]. Noteworthy, however, the role of the MAPK signaling pathways in steroidogenesis is poorly understood as a

MAP kinase	Other names	Relevant information	Phosphorylation site motif
ERK1	P44 MAPK	~86% sequence similarity to ERK2, ubiquitously expressed, UO126 sensitive	TEY
ERK2	P42 MAPK	Ubiquitously expressed, UO126 sensitive	TEY
ERK3	P63, ERKα	Yields a single protein of 100 kDa possessing a C-terminal extension	SEG
ERK1b	ERK4	A separate gene	TEY
JNK1	SAPKy	Several splice variants	TPY
JNK2	SAPKa	Several splice variants	TYP
JNK3	$SAPK\beta$	Several splice variants	TYP
Ρ38α	P38, CSBP, SAPK2, MAPK14	Sensitive to SB203580	TGY
Ρ38β	P38-2, MAPK11	Sensitive to SB203580	TGY
Ρ38γ	ERK6, SAPK3, MAPK12	SB203580 insensitive	TGY
Ρ38δ	SAPK4 or MAPK13	SB203580 insensitive	TGY
Mxi2		A splice form of P38 α	TGY
Exip		A splice form of P38 α	TGY
ERK5	BMK1	Cell proliferation, differentiation	TEY
ERK7	MAPK15	Cell proliferation	TEY
NLK	Nemo-like kinase	Ortholog of <i>C. elegans</i> LIT-1, relative of Drosophila nemo	TQE
MAK	Male germ cell- associated kinase	Expressed in testicular cells undergoing meiosis	TDY
MRK	MAK-related kinase	Ubiquitous in adult tissues	TDY
MOK		Phorbol ester sensitive	TEY
KKIALRE		Cdc2-related kinase	TDY
KKIAMRE		T, Y mutants activated in cells	TDY

TABLE 1: Mammalian MAP kinases.

consequence of conflicting reports demonstrating stimulation, inhibition, or no effect in different steroidogenic cells [12, 15–18].

The steroidogenic acute regulatory protein (StAR) mediates the rate-limiting and regulated step in steroid hormone biosynthesis, that is, the transfer of the substrate for all steroid hormones, cholesterol, from the outer to the inner mitochondrial membrane [19-25]. As such, this protein plays a crucial role in the regulation of steroid hormones required for life itself, in the case of adrenal steroids, and for maintaining normal reproductive function, in the case of gonadal steroids. StAR is a rapidly synthesized, labile phosphoprotein, whose expression, activation and extinction are regulated by PKA, PKC, and a host of other signaling pathways (reviewed in [23, 25-27]). The StAR protein is localized to the mitochondria and consists of several forms of a newly synthesized 30-kDa protein, which has a 37 kDa precursor form containing an N-terminal mitochondrial targeting sequence [28, 29]. StAR is primarily associated with steroid producing tissues, and studies have demonstrated a tight correlation between the synthesis of StAR proteins and the synthesis of steroids. The compelling evidence for the critical role StAR in the regulation of steroidogenesis has been illustrated by the targeted disruption of the StAR gene and by the study of patients suffering from lipoid

congenital adrenal hyperplasia, in which both adrenal and gonadal steroid biosyntheses are severely impaired due to mutations in the StAR gene [30-33]. In the adrenal and gonads, cAMP mediated mechanisms predominantly regulate expression of the StAR protein and steroid synthesis that involve transcriptional and translational induction. Conversely, transcriptional and/or translational inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis, with the exception of approximately 10%-15% of steroid production that appears to occur through StAR-independent events [34-38]. It should be noted that phosphorylation of StAR is required to produce optimal cholesterol transferring ability of the StAR protein in steroid biosynthesis. Two putative PKA phosphorylation sites at serine 56/57 and serine194/195 have been identified, in murine and human StAR respectively, and mutations in these sites demonstrated the functional importance of the latter in the biological activity of StAR [39, 40]. While the cAMP/PKA pathway is unquestionably the major signaling pathway for trophic hormone-stimulated StAR expression and steroid biosynthesis, it has been well established that the MAPK signaling cascades play important roles in regulating these processes. The purpose of this paper is to summarize the findings of a number of laboratories, including our own, that have examined the roles of four distinct mammalian MAPK cascades in regulating steroidogenesis, and where evidence exists, on StAR expression, in steroidogenic cells.

2. The MAPK Signaling Cascade

The MAPK signaling cascade involves activations of several transmembrane signaling molecules, cytoplasmic protein kinases, and a network of interacting proteins, and these, in turn, regulate almost all cellular processes, from gene expression to cell death [1, 3, 7, 14, 41, 42]. Each of these cascades consists of several tiers of protein kinases that sequentially activate each other upon phosphorylation. Activation of each of the MAPK pathways is mediated by small GTPbinding proteins (e.g., Ras, Rap, or other oncoproteins), or by scaffold proteins or by adaptor molecules [41, 43, 44]. These events allow the transmission of extracellular signals to appropriate intracellular targets either directly or through three-tiered kinase modules [8, 10, 41]. MAPKs are activated by phosphorylation cascades that contain at least two upstream protein kinases. In all MAPK cascades, the kinase immediately upstreaming of the MAP kinase is a member of the MAP/ERK kinase (MEK or MKK) family. These are dual-specificity enzymes that are capable of phosphorylating serine/threonine and tyrosine residues in their MAP kinase substrates in order to activate the protein's activity [8, 45–47]. However, it is known that MAP kinases have overlapping substrate specificities. The MEKs are also activated by phosphorylation on serine or threonine residues in their activation loops. There are several and diverse MEK kinases (MEKKs) that activate MEKs [4, 9]. In contrast, the activity of MAPKs is negatively regulated by MAPK phosphatases, a group of dual-specificity phosphatases that remove phosphate from serine/threonine or tyrosine residues and thereby inactivate MAPKs for controlling signals [48-50].

The MAPK signaling pathway has long been implicated in the regulation of cell cycle progression, is used as a biochemical marker in evaluating the mitogenic effects of a variety of stimuli, and is a point of convergence for diverse signaling pathways [12, 15, 16, 51, 52]. In mammals, four distinct MAPK cascades (ERK1/2, p38, JNK/SAPK, and ERK5/BMK1) are primarily activated by specific MAPKKs, that is, MEK1 and MEK2 for ERK1/2, MKK3/6 for p38, MKK4/7 for JNK/SAPK, and MEK5 for ERK5/BMK1 [1, 2, 41]. Nevertheless, each MAPKK can be induced by many MAPKKKs, and presumably each MAPKKK confers responsiveness to discrete signals [9, 53]. The differential expression, activation, and substrate specificity of these MAPKs suggest their varying physiological functions in different cellular contexts. A substantial body of evidence indicates that MAPK signaling cascades are activated by a large number of extracellular signals and play pivotal roles in regulating the steroidogenic response. As such, a variety of exogenous stimuli (e.g., hormones, growth factors, and cytokines) that influence different MAPK cascades exhibit diverse effects on StAR expression and steroidogenesis in different steroidogenic cell models (Figure 1; [12, 15-17, 54, 55]).

2.1. ERK1/2 Signaling and Its Role in StAR Expression and Steroidogenesis. The ERK1/2 signaling cascade is the most widely studied member of the MAPKs. ERK1 (p44 MAPK) and ERK2 (p42 MAPK) are highly conserved throughout eukaryotic cells and bring together transmembrane receptors and a network of various proteins and integrating signals to control many cellular processes, including cell survival, differentiation, tumor progression, and invasion [4, 7, 56-58]. There are also alternatively spliced forms of ERKs, namely, ERK1b (46 kDa), ERK1c (42 kDa), and ERK2b (41 kDa). ERKs are ubiquitously expressed to varying extents in different tissues. Both ERK1 and ERK2 are activated by numerous extracellular stimuli, including growth factors, cytokines, transforming agents, and ligands that act via G protein-coupled receptors (GPCRs) and tyrosine kinase receptors. The two phosphoacceptor sites, tyrosine and threonine, in the activation loop (TEY) are phosphorylated in order to activate the ERK1/2 signaling cascade, which occurs exclusively through MEK1 and MEK2. ERKs also phosphorylate several substrates (e.g., ribosomal S6 kinase (RSK), the MAPK/SAPK-activated kinase (MSK)) either in the cytosol (e.g., PLA2) or in the nucleus (e.g., Elk1) [1, 4, 9, 11]. However, this activation requires adaptor proteins that are linked to the guanine exchange factors (GEFs) of GTPbinding proteins. Following stimulation, recruitment of the adaptor protein-GEF complex to the plasma membrane results in the induction of Ras or related molecules, which then transmit signals to the MAP3K level of the cascade (Raf-1, B-Raf, A-Raf, TLP-2, and MEKK1) [1, 9, 59]. This, however, is an oversimplification of the ERK1/2 signaling module. Generally, the three-tiered system of the ERK1/2 signaling cascade is rather complex and involves many MEKKs that are capable of phosphorylating a number of MEKs and thereby activating several MAP kinases and have been discussed elsewhere [1, 2, 4, 11, 41].

The involvement of the ERK1/2 pathway in steroidogenesis has been widely studied. This signaling pathway is involved in regulating StAR expression and steroid biosynthesis, but several seeming contradictions have been reported in different steroidogenic tissues [12, 15-17, 54, 60-62]. For example, it has been demonstrated that activation of ERK1/2 by hCG increases StAR expression and testosterone production while inhibition of ERK1/2 by U0126 results in decreases in these parameters in hCG-stimulated primary cultures of immature rat Leydig cells [61]. In contrast, ERK1/2 inhibition enhances StAR expression as induced by LH, insulin-like growth factor-1 (IGF-1), transforming growth factor- α , and interleukin-1 α (IL-1 α) but decreases steroid levels [2, 12, 52, 62, 63]. It has been reported that steroid biosynthesis induced by hCG and fibroblast growth factor-9 (FGF-9) is mediated through the involvement of Ras-MAPK and PKA signaling in mouse Leydig cells [64, 65]. We have demonstrated that the ERK1/2 signaling cascade plays key roles in PKA-mediated (activated by (Bu)₂cAMP and hCG)- and PKC-mediated (activated by PMA) regulation of steroidogenesis [12, 52, 66]. All these agents activate ERK1/2; however, they have diverse effects on StAR expression and steroid synthesis in conjunction with ERK1/2 inhibition by either PD98059 or U0126. In particular, whereas



FIGURE 1: A schematic model illustrating the involvement of multiple signaling in MAPK-mediated regulation of StAR expression and steroidogenesis. Interaction of trophic hormones with their specific membrane receptors results in the activation of G proteins (G), which, in turn, stimulate adenylate cyclase (AC) that catalyzes the production of cAMP from ATP. cAMP then activates PKA and results in the phosphorylation of transcription factors involved in StAR gene transcription. The binding of growth factors results in activation of receptor tyrosine kinase and mediates biological functions via a number of mechanisms, including receptor autophosphorylation, receptor clustering, and phosphorylation of intracellular proteins. This leads to the activation of a cascade of protein kinases including Ras/Raf, and other related kinases. These protein kinases, in turn, activate different transcription factors, including CREB/ATF-1, cFos, and cJun. Phosphorylation of these transcription factors results in the transcriptional regulation of the StAR gene and, thus, steroid biosynthesis. The PKA and PKC signaling pathways can directly or indirectly activate transcription factors are also capable of activating a cascade of protein kinases (Rap/Ras/Raf or other related oncoproteins) leading to a number of MAPK signaling cascades, which have been demonstrated to play important roles in regulating StAR expression and steroid biosynthesis in steroidogeneic tissues.

the inhibition of ERK1/2 increases cAMP/hCG-stimulated StAR expression, it decreases PMA-mediated StAR levels. Nonetheless, progesterone levels were diminished in all cases. The decrease in steroid synthesis was not associated with attenuation of the cytochrome P450 side chain cleavage (P450scc) and 3β -hydroxysteroid dehydrogenase (3β -HSD) enzyme activities, as ERK1/2 inhibition had no effect on 22R-hydroxycholesterol-mediated steroid synthesis [12]. Note-worthy, the increases in PMA, (Bu)₂cAMP, and hCG-induced StAR expression and progesterone levels were inversely correlated with the levels of a negative transcription factor,

namely, dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X-chromosome, gene 1 (DAX-1) [12, 52, 66, 67]. While U0126 attenuates DAX-1 expression, this inhibition can be reversed by PMA but not by (Bu)₂cAMP/hCG [12, 66]. In fact, the differential effects of U0126 on PKA- and PKC-mediated steroidogenesis is mediated, at least in part, by alterations in DAX-1 expression in mouse Leydig cells. Furthermore, studies have shown that both (Bu)₂cAMP and PMA can elevate the levels of scavenger receptor class B type 1 (SR-B1, a high-density lipoprotein (HDL) receptor that binds various lipoprotein particles

and facilitates cellular cholesterol influx) and that ERK1/2 inhibition decreases SR-B1 expression [12]. The binding of HDL to SR-B1 has been reported to stimulate the ERK1/2 pathway following Ras activation [68]. SR-BI is involved in importing cholesterol into cells that will eventually be provided to the mitochondria for steroid biosynthesis [52, 69]. Thus it is plausible that the decrease in steroid synthesis caused by U0126 (during conditions of elevated StAR) is due to a reduction in cholesterol availability to the mitochondria. Recent studies have shown that activation of ERK1/2 at the mitochondria plays an important role in Leydig cell steroidogenesis [70, 71]. Specifically, ERK1/2 is capable of phosphorylating StAR at serine 232 in the presence of cholesterol, demonstrating that StAR is a substrate for ERK1/2 and that a mitochondrial kinase complex is essential for maximum cholesterol transferring capacity of the StAR protein in steroid synthesis.

In granulosa cell-derived rLHR-4 and rFSHR-17 cell lines, treatments with LH/hCG and FSH, respectively, increase ERK1/2 phosphorylation, StAR expression, and progesterone synthesis [16, 17, 51, 72]. Conversely, gonadotropin-stimulated StAR and steroid levels have been shown to be further augmented following inhibition of ERK1/2 both by PD98059 and U0126, suggesting that the induction of LH/hCG- and FSH-induced steroidogenesis involves downregulation of the steroidogenic machinery including the ERK1/2 cascade. Studies have also shown that while PD98059 and U0126 have no effects on LH/hCG, insulin, or IGF-1-induced steroidogenesis, they decrease (Bu)₂cAMP, cholera toxin and forskolin stimulated progesterone production [54, 60]. Further, it has been reported that FGF-8 mediated ERK1/2 activity is associated with an attenuation of estradiol production in rat granulosa cells [73]. Also, ERK1/2 activation results in differential effects on LH-induced StAR and Cyp17 expression in bovine theca cells. Activation results in an increase in StAR and a decrease in Cyp17 that in turn results in an elevation in progesterone and a reduction in androsterone levels, processes in which DAX-1 and steroidogenic factor 1 (SF-1) play pivotal roles [17]. Likewise, administration of hCG in vivo or treatment with gonadotropins/cAMP in vitro is associated with down-regulation of Cyp19a1 and upregulation of StAR and Cyp11a1 mRNA expression in both mural and cumulus granulosa cells of mouse preovulatory follicles [74]. These events have been shown to be tightly regulated with steroid biosynthesis and involve ERK1/2-dependent signaling. Recently, the activation of ERK1/2, as well as other MAPK signaling, by leptin, has been linked to decreases in basal and cAMP/PKA stimulated StAR expression and progesterone synthesis without affecting the levels of the P450scc and 3β -HSD enzymes [75]. Additionally, treatment with metformin (a drug that is widely used for treating infertility in women with polycystic ovary syndrome (PCOS)) causes an activation of ERK1/2 that results in suppression of insulin-stimulated P450 aromatase mRNA expression and activity [76]. In theca cells isolated from PCOS patients, increases in Cyp17 expression and androgen biosynthesis are connected to an attenuation of ERK1/2/MEK1/2 activity by PD98059, suggesting the involvement of MAPK signaling

in the pathogenesis of this disease [77]. Prostaglandin $F_{2\alpha}$ (PGF_{2 α}), an agent that influences luteal regression through the induction of the phospholipase C/diacylglycerol/PKC pathway, induces phosphorylation of ERK1/2, which, in contrast, decreases basal and LH/hCG-stimulated StAR expression and progesterone synthesis in human granulosa-luteal cells [78]. These effects of PGF_{2 α} in the steroidogenic response can be reversed following inhibition of MEK activity by PD98059. Moreover, the inhibitory effect of PGF_{2 α} on StAR expression and steroidogenesis, in rat luteal cells, is modulated by the negatively acting transcription factors, DAX-1 and ying yang 1 [18, 79]. These results demonstrate the multiple effects of the ERK1/2 signaling cascade in StAR expression and steroidogenesis in gonadal cells.

In mouse Y-1 adrenocortical tumor cells the induction of forskolin-mediated corticosterone synthesis is dependent upon the activation of ERK1/2 and is correlated with phosphorylation of SF-1 and increased StAR gene transcription [15]. Accordingly, inhibition of ERK1/2, by either PD98059 or U0126, decreases StAR expression and steroid production without affecting P450scc enzyme activity. Increasing evidence demonstrates that ACTH and angiotensin II (Ang II) can elevate phosphorylation of p44/p42 MAPKs and result in increases in StAR expression and steroid synthesis that involve PKC signaling and Ras/Raf-1 kinase [80-83]. The induction of orexins A and B (neuropeptide hormones) mediated StAR expression and steroid synthesis is mediated by a number of MAPK cascades including EKR1/2 activation, where the latter requires multiple Gprotein signaling pathways in human H295R adrenocortical cells [55, 84]. It has been demonstrated that while proopiomelanocortin (POMC) fragments, 1-28-POMC and 1-48-POMC, modulate cellular proliferation, they decrease adrenal steroidogenesis by activating ERK1/2 signaling [85]. Chronic administration of ACTH induces the phosphorylation of ERK1/2 that occurs in parallel with adrenal corticosterone synthesis in adult rats [82]. In both H295R and primary cultures of human adrenocortical cells, treatment with adipokines up-regulates the ERK1/2 cascade and results in increases in StAR expression and aldosterone synthesis, processes that do not require cAMP/PKA signaling [86, 87]. Taken together, it seems clear that the ERK1/2 signaling cascade plays diverse roles in regulating StAR expression and steroidogenesis. These roles could be a result of the existence of multiple signal transduction pathways that display differences in receptor-effector coupling between tissues and species.

2.2. p38 MAPK Signaling and Its Role in Steroidogenesis. The p38 MAPK signaling cascade is thought to participate in the response of cells to stress. Four members of the p38 MAPK family have been cloned and named p38 α (MAPK14), p38 β (p38-2), p38 γ (ERK6 or SAPK3), and p38 δ (SAPK4) and share approximately 60% homology in their amino acid sequences [10, 88–91]. Also, several alternatively spliced isoforms of p38 MAPK include Mxi2 (identical to p38 α) and Exip. P38 MAPKs contain a Thr-Gly-Tyr activation loop sequence (TGY). They are activated by dual phosphorylations on threonine and tyrosine residues in response to numerous stimuli, including cytokines, hormones, GPCRs, heat shock, and other stresses and play important roles in controlling many cellular functions [42, 53, 92, 93]. P38 α is expressed in most cells; however, expression of other isoforms is tissue specific. Cellular distribution, activation, and substrate specificity of p38 MAPKs result in diverse biological functions. Once activated, p38 MAPKs either transmit the signals via a three-tier cascade or phosphorylate other regulatory molecules such as PLA2, heat shock proteins, and transcriptions factors (c-Jun, ATF-2, CREB, CHOP, NF-kB, and others) [8, 42, 92, 94]. Substrates of p38 MAPK include MAPK-activated protein kinases (MKs), that is, MK2, MK3, and MK5 (reviewed in [1, 53, 95, 96]). There are also several distinct kinases at the MAP3K level of the p38 MAPK cascade, including MLK2, MLK3, TPL2, dual leucine zipper-bearing kinase, ASK1, MAP three kinase 1, and TAK1 [91, 97, 98]. Studies have reported the existence of p38 signaling crosstalk with other MAPK cascades. For instance, the p38 MAPK pathway causes rapid inactivation of the ERK1/2 cascade mediated by PP2A [99]. The p38 MAPK pathway is involved in tissue homeostasis and several pathologies ranging from inflammation and the immune response to cancer and neurodegenerative diseases [93, 100, 101].

The p38 MAPK signaling cascade has been implicated in regulating steroidogenesis. It has been demonstrated that IL-1 α activates p38 MAPK and that this event is associated with StAR expression and testosterone synthesis in immature rat Leydig cells [102, 103]. The involvement of p38 MAPK in steroidogenesis is further assessed by observations in which inhibition of its activity, either by SB203580 or PD169316, results in the coordinate suppression of StAR and steroid levels. IL-1 α is also capable of phosphorylating CREB and Fos/Jun through the activation of a p38 substrate which is a RSK family member (RSK-B kinase, also called MSK2), suggesting that this process may play a role in the differentiation of immature into adult Leydig cells [102]. In addition, it is worth noting that IL-1 α also activates ERK1/2 and inhibition of the latter by U0126 augments expression and phosphorylation of the StAR protein but decreases androgen synthesis by dissipating the mitochondrial electrochemical potential [63]. These findings suggest that a number of MAPK signaling events differentially influence IL- 1α -mediated steroidogenesis in mouse Leydig cells.

Gonadotropins have been shown to activate both p38 and ERK1/2 MAPKs and result in varying effects on StAR expression and steroidogenesis in ovarian granulosa cells [16, 17, 51, 104]. Studies have demonstrated that interference of p38 (by SB203580) and ERK1/2 (by PD98059 and U0126) activity is associated with increases in LH/hCG/FSH mediated StAR expression and progesterone synthesis [16, 51, 104]. In addition, inhibition of p38 decreases both P450arom and estradiol synthesis, and these events are tightly correlated with liver receptor homolog-1 and DAX-1 expression [104], demonstrating that p38 targets these transcription factors in regulating steroidogenesis. Heat shock protein HSP-27 is identified as a downstream phosphorylation target of FSH- and forskolin-mediated p38 MAPK activation [105]. In granulosa cell-oocyte cocultures, both bone morphogenetic protein-2 (BMP-2) and BMP-4 exert differential effects on FSH mediated regulation of steroidogenesis through the activation of p38 MAPK [106]. Indeed, both BMP-2 and - 4 increase FSH-mediated P450arom mRNA expression and estradiol production but decrease StAR and progesterone levels.

In primary cultures of rat adrenal glomerulosa cells, Ang II activates the p38 and ERK1/2 signaling pathways and results in increases in StAR expression, steroidogenic enzymes, and steroid synthesis [83, 107]. Concurrently, Ang II inhibits protein synthesis by enhancing p27kip1 expression (a protein known to block the cell cycle in the G1 phase). The effects of Ang II can be reversed through inhibition of p38 activity by SB203580, suggesting that Ang II plays an important role in adrenal physiology. Accumulating evidence indicates that activation of the p38 MAPK signaling cascade is linked to the aging-induced, oxidative stressmediated suppression of steroidogenesis in adrenal cells [108-110]. Alternatively, inhibitors of p38 MAPKs (by either SB203580 or SB202190) and antioxidants (reactive oxygen species (ROS) scavengers MnTMPyP and N-acetyl cysteine) have been shown to restore corticosterone synthesis in cells from aged rats. These findings indicate that the stress-mediated inhibition of steroid biosynthesis involves the activation of the p38 MAPK pathway in the adrenals during the course of aging [110]. Also, intense phospho-p38 MAPK immunoreactivity has been detected in human brains of postmortem patients afflicted with Alzheimer's disease [111, 112], indicating that p38 could be involved in the pathogenesis of this disease.

2.3. JNK/SAPK Signaling and Its Role in Steroidogenesis. JNK/SAPKs are also considered as stress-activated MAPKs; however, they are different from p38 MAPKs [1, 113, 114]. Isolation and subsequent characterization of cDNAs encoding these enzymes revealed three genes encoding proteins with 10 or more alternatively spliced forms. Three main isoforms, that is, JNK1/SAPKγ (46 kDa), JNK2/SAPKα (54 kDa), and JNK3/SAPK β (52 kDa), are approximately 85% identical in their core catalytic domains [115, 116]. Whereas JNK1/2 MAPKs are ubiquitously expressed, JNK3 is primarily localized to neuronal tissues, testis and cardiac myocytes. JNK/SAPKs are activated by cytokines, UV irradiation, growth-factor deprivation, agents that interfere with DNA and protein synthesis, as well as other stressors. Similar to other MAP kinases, the activity of JNK/SAPKs is dependent upon phosphorylation on tyrosine and threonine residues, which are separated by a proline to generate the TPY motif in the activation loop. The resulting signals are then transmitted to three-tier cascades either directly or through MAP4Ks [117, 118]. Several kinases are phosphorylated in these cascades [1, 41]. However, two MEK family members, MKK4 (SEK1, MEK4, JNKK1, and SKK1) and MKK7 (MEK7, JNKK2, and SKK4), are predominantly involved in the JNK/SAPK cascade for signal integration [117–119].

Several lines of evidence demonstrate the involvement of the JNK/SAPK signaling cascade in steroidogenesis. For example, tumor necrosis factor- α (TNF α) activates JNK/SAPK; however, the latter is associated with decreases in basal and cAMP-induced steroidogenesis by reducing Cyp17 gene expression in MA-10 cells [120]. TNF α also decreases the phosphorylation of ERK1/2 while simultaneously increasing the abundance of cJun as well as increasing AP-1 binding activity, suggesting the involvement of a number of MAPKs in TNFα signaling. Therefore, the activation of JNK/SAPK and ERK1/2 MAPKs appears to play a mutually antagonistic role in TNFa-mediated steroidogenesis. In rat R2C Leydig tumor cells, bisphenol A (BPA), an endocrine disruptor, is capable of activating JNK/SAPK and results in an elevation in aromatase activity and an attenuation of testosterone synthesis by targeting both CREB and Akt [121]. Increasing evidence demonstrates that steroidogenesis decreases in aging, a time when the levels of reactive oxygen species (ROS) increase. As a consequence, a number of ROS, such as superoxide anion (O_2^{-}) and hydrogen peroxide (H_2O_2) , have been involved in the repression of testicular StAR expression and steroid synthesis [122–124]. In K28 mouse Leydig cells, the inhibition of ROS-mediated StAR, P450c17 mRNA, and steroid levels is mediated, at least in part, through the activation of JNK/SAPK MAPKs and subsequent upregulation of c-Jun [124]. These events cause a repression in the trans-activation potential of Nur77 on steroidogenic enzyme genes and result in decreases in StAR expression and steroidogenesis.

In human granulosa cells, leptin activates a number of MAPKs including the JNK/SAPK signaling pathway where it decreases cAMP-induced StAR protein expression and progesterone synthesis [75]. Likewise, BMPs can induce phosphorylation of several MAPK cascades, exhibit varying actions on steroidogenesis, and play important roles in ovarian follicular growth and maturation [73, 125, 126]. Also, an oocyte derived factor, FGF-8, has been shown to interact with BMPs, activate the JNK/SAPK and ERK1/2 signaling cascades and subsequently regulate FSH-induced steroidogenesis in a rat granulosa cell-oocyte coculture system [73]. Altogether, the JNK/SAPK signaling cascade is fundamentally connected with stress-related responses and the activation of JNK/SAPK decreases the steroidogenic response in a number of steroidogenic tissues.

2.4. ERK5/BMK1 Signaling and Its Role in Steroidogenesis. The ERK5/BMK1 is the largest known MAP kinase (~110 kDa) family member. The signaling pathway leading to ERK5 activation is poorly understood in comparison with other MAPKs as a consequence of conflicting findings in the literature [127–130]. Importantly, the C-terminus of ERK5 contains 10 consensus MAP kinase phosphorylation sites which can be autophosphorylated [131]. ERK5 is ubiquitously expressed and its activity is regulated by a variety of proliferative (growth factors, phorbol ester, serum, and lysophosphatidic acid) and cell-stressing (H₂O₂, sorbitol, and UV irradiation) agents [132, 133]. The mechanism of upstream activation of the ERK5 cascade has not been fully defined. This mechanism may include the action of adaptor proteins (e.g., LAD), protein tyrosine kinases, and WNK1 [134–136]. These components have been shown to induce a number of kinases at the level of MAP3K, including MEKK2/3, TPL2, and MLTK [135, 137-139]. These kinases then phosphorylate MEK5 (an upstream component of ERK5) on serine and threonine residues [127]. MEK5 then activates ERK5 on threonine and tyrosine residues in the loop sequence motif (TEY) identical to ERK1 and ERK2. However, ERK5 cannot be phosphorylated by MEK1/2, and MEK5 does not phosphorylate ERK1/2. Another substrate of ERK5 is the serum and glucocorticoid-inducible kinase, which may serve as a MAPKAPK of this cascade, allowing for the possible involvement of a five-tier cascade [140]. ERK5 can affect a number of cellular activities (e.g., cellular proliferation, differentiation, and motility) by phosphorylating many transcription factors, including MADs box, c-Myc, c-Fos, myocyte enhancer factors 2A and C, and SAP1a [133, 141–143].

Relatively little is known regarding the role of ERK5 in steroidogenesis. Recently, it has been reported that orexins A and B activate ERK5 and concomitantly increase expression of the StAR protein and steroidogenic enzyme genes in H295R cells [55, 84]. These agents can also augment cortisol secretion in primary cultures of adrenal cells. The effects of orexins on ERK5 phosphorylation have been demonstrated to be similar to those of Ang II and are mediated by multiple G-protein signaling pathways. However, orexins simultaneously activate the ERK1/2 and p38 MAPK signaling cascades [55], indicating that the regulation of orexin-mediated StAR expression and steroidogenesis involves several MAPKs in adrenal cells. Information that the activation of the ERK5 signaling pathway in gonadal cells is linked to steroidogenesis is currently lacking.

3. Other MAP Kinases

ERK3, ERK4, ERK7, NLK, MOK, and others are considered as atypical MAPKs (Table 1). Regulation, structure, and substrate specificity of these MAPKs have been described in a recent review [5]. The roles of these atypical MAPKs in steroidogenesis remain to be elucidated.

4. Conclusions

The MAPK signaling cascades are the major components of pathways controlling a wide variety of cellular processes, including embryogenesis, gene expression, acute responses to hormones, cell survival, and apoptosis and are of critical importance in the transduction of extracellular signals in cells. Noteworthy, the MAPK cascade has been implicated in the pathogenesis of a number of human disorders, including Alzheimer's disease, Parkinson's disease, and many cancers. Indeed, the multiple effects of MAPKs could be a result of the involvement of a wide variety of substrates that include protein kinases and phosphatases, transcription factors, cytoskeletal elements, and other signal-regulated molecules. The studies summarized here have emphasized the roles of ERK1/2, p38 MAPK, JNK/SAPK, and ERK5 MAPKs in the regulation of StAR expression and steroidogenesis in different steroidogenic tissues. The StAR protein plays an indispensable role in the production of steroid hormones required for bodily homeostasis and normal reproductive development and function. Based on the results available at this time, it is obvious that the MAPK signaling cascades play diverse roles in controlling StAR expression and steroid biosynthesis in tissue-, stimulus-, and pathwayspecific manners. These processes appear to be dependent on receptor-effector coupling, signaling crosstalk, and/or other factor(s) involved in steroidogenesis. Moreover, under specific circumstances, the regulation of MAPK-dependent StAR expression and steroidogenesis involves more than one MAPK signaling, and as a consequence different, and even opposing, effects of MAPKs can be seen in different steroidogenic cells. Additionally, different cells express distinct sets of transcription factors, and this diversity may account for the cell-type-dependent specificity of MAPK action. Given the physiological and pathological roles of the MAPK signaling pathways, elucidation of tissue- and diseasespecific effects of each of the MAPK signaling cascades together with their downstream effectors requires better understanding. An abundance of molecular tools including high throughput genomic and proteomic technologies will undoubtedly provide valuable insights into these regulatory mechanisms.

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Review Article

Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways?

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Mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases that play the major role in signal transduction from the cell surface to the nucleus. MAPKs, which consist of growth factor-regulated extracellular signal-related kinases (ERKs), and the stress-activated MAPKs, c-jun NH₂-terminal kinases (JNKs) and p38 MAPKs, are part of a three-kinase signaling module composed of the MAPK, an MAPK kinase (MAP2K) and an MAPK kinase (MAP3K). MAP3Ks phosphorylate MAP2Ks, which in turn activate MAPKs. MAPK phosphatases (MKPs), which recognize the TXY amino acid motif present in MAPKs, dephosphorylate and deactivate MAPKs. MAPK pathways are known to be influenced not only by receptor ligand interactions, but also by different stressors placed on the cell. One type of stress that induces potential activation of MAPK pathways is the oxidative stress caused by reactive oxygen species (ROS). Generally, increased ROS production in a cell leads to the activation of ERKs, JNKs, or p38 MAPKs, but the mechanisms by which ROS can activate these kinases are unclear. Oxidative modifications of MAPK signaling proteins and inactivation and/or degradation of MKPs may provide the plausible mechanisms for activation of MAPK pathways by ROS, which will be reviewed in this paper.

1. Introduction

Mitogen-activated protein kinases (MAPKs) compose a family of protein kinases that play an essential role in relaying extracellular signals from the cell membrane to the nucleus *via* a cascade of phosphorylation events and are negatively regulated by MAPK phosphatases (MKPs) [1]. Diverse cellular functions, ranging from cell survival to cell death, are regulated by MAPK signaling [2]. A number of extracellular and intracellular stimuli have been shown to activate MAPK pathways at cellular levels [3], implying that there may be tight and specific regulation of MAPK activation by a certain stimulus. Interestingly, reactive oxygen

species (ROS) can activate MAPK pathways [4], but the mechanism(s) for this effect is unclear.

Besides MAPKs, other signaling molecules (e.g., protein tyrosine phosphatases, protein tyrosine kinases, and transcriptional factors) can also be activated by ROS [5], suggesting that ROS may have meaningful roles as regulators of cell function or as signaling molecules. Indeed, mounting evidence supports a physiological role for ROS as a "second messenger" in intracellular signaling cascades that control cell growth, proliferation, migration, and apoptosis [5].

Because the MAPK pathways mediate both mitogen- and stress-activated signals, there has been significant interest in the regulation of these pathways by ROS. This paper will
focus on the putative mechanisms by which ROS can activate MAPK pathways in a cell.

2. ROS

ROS include superoxide anion radical $(\cdot O_2^{-})$, hydroxyl radicals $(\cdot OH)$, and hydrogen peroxide (H_2O_2) . H_2O_2 is not a free radical and a weaker oxidizing agent than the free radical $\cdot O_2^{-}$. However, in the presence of transition metals such as iron or copper, H_2O_2 can be oxidized into the extremely reactive and toxic $\cdot OH$ *via* well-known Fenton reaction. In the cellular systems, ROS are normally counteracted by ubiquitously expressed antioxidant proteins, such as superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase, thioredoxin, glutaredoxin, and GSH. For example, SOD can convert $\cdot O_2^{-}$ into H_2O_2 , whereas catalase and GSH peroxidase can reduce H_2O_2 .

ROS are constantly produced by a number of normal cellular events, with a major source being aerobic respiration, but ROS produced during these events are generally counteracted by several antioxidant proteins [6, 7]. A large amount of ROS can also be produced by inflammatory processes, ionizing radiation, and many chemotherapeutic drugs, and this, if the production of ROS exceeds the capacity of the antioxidant proteins, may cause the so-called "oxidative stress"; in a biological sense, the oxidative stress may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury [5, 7].

Oxidative stress is known to be implicated in many human diseases, including atherosclerosis, cancer, neurodegenerative diseases, and aging [7]. However, there is still a debate whether oxidative stress is a cause or a result of these diseases, largely due to a lack of our understanding of the mechanisms by which ROS function in both normal physiological and disease states.

ROS are not only injurious to cell survival but also essential to cell signaling and regulation, and this may be dependent on the levels of produced ROS. At high levels, ROS can lead to impaired physiological function through cellular damage of DNA, proteins, phospholipids, and other macromolecules, which can lead to certain human pathologies [8]. At low levels, ROS can alter intracellular redox state, leading to activation of redox-sensitive proteins, and also modify redox-sensitive parts of proteins, potentially inhibiting or increasing their enzymatic activity [9, 10]. H_2O_2 , with a relatively long half-life, good membrane permeability, and higher intracellular concentration, has been proposed to function as a second messenger [9, 10]. In this regard, it is most likely that H_2O_2 may mimic many actions of ROS in a cellular system.

3. MAPKs

The MAPKs comprise a family of ubiquitous prolinedirected, protein-serine/threonine kinases, which play an essential role in sequential transduction of biological signals from the cell membrane to the nucleus [11]. In mammalian cells, there are three well-defined subgroups of MAPKs:



FIGURE 1: MAPK cascades. MAPK signaling pathways mediate intracellular signaling initiated by extracellular or intracellular stimuli. MAP3Ks phosphorylate MAP2Ks, which in turn phosphorylate MAPKs. Activated MAPKs phosphorylate various substrate proteins (e.g., transcription factors), resulting in regulation of various cellular activities (e.g., proliferation, differentiation, inflammatory responses, and apoptosis). Activation by MAPK signaling cascades is achieved either through a series of binary interactions among the kinase components or through formation of a multiple kinase complex.

the extracellular signal regulated kinases (ERKs, including ERK-1 and ERK-2 isoforms), the c-Jun N-terminal kinases (JNKs, including JNK-1, JNK-2, and JNK-3 isoforms), and the p38 MAPKs (including p38- α , p38- β , p38- γ , and p38- δ isoforms). Each subgroup of MAPKs is activated through a cascade of sequential phosphorylation events, beginning with the activation of MAPK kinase kinases (MAP3Ks). The MAP3Ks phosphorylate and activate a downstream dualspecificity MAPK kinases (MAP2Ks), which in turn stimulate MAPK activity through dual phosphorylation on threonine and tyrosine residues within a conserved tripeptide motif [1, 11]. The well-defined regulation of MAPK signaling pathways is summarized in Figure 1. It should be noted that the three subgroups of MAPKs (i.e., ERKs, JNKs, and p38 MAPKs) are involved in both cell growth and cell death, and the tight regulation of these pathways is paramount in determining cell fate [12]. The deleterious consequences of sustained activation of MAPK pathways may include excessive production of MAPK-regulated genes, uncontrolled proliferation, and unscheduled cell death.

3.1. ERKs. ERK pathway is activated by MAP/ERK Kinase (MEK), which is activated by Raf. Raf, an MAP3K, is activated by the Ras-GTPase, whose activation is induced by receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) receptor [13]. Growth factor receptors are most commonly activated by ligand-induced dimerization or oligomerization that phosphorylates RTKs [14]. Ligand-independent clustering and activation of growth factor receptors in response to ROS have also been well demonstrated [15]. Meves et al. [16] demonstrated that

oxidative stress induces EGF receptor activation through RTK phosphorylation and proposed that H_2O_2 is a critical mediator required for ligand-independent phosphorylation of growth factor receptors in response to oxidative stress.

3.2. p38 MAPKs. The p38 MAPKs are usually activated in response to inflammatory cytokines, as well as by many other stimuli, including hormones, ligands for G proteincoupled receptors, and stresses such as heat shock and osmotic shock [17]. Two MEK family members, MEK3 (or MKK3) and MEK6 (or MKK6), are highly specific for p38 MAPKs [17]. MKK6 can phosphorylate the four p38 MAPK family members, while MKK3 phosphorylates $p38\alpha$, p38y, and p38 δ , but not p38 β . Both will also phosphorylate JNK isoforms [17]. Several MAP3Ks have been shown to trigger p38 MAPK activation, and they include ASK1 (apoptosis signal-regulating kinase 1), DLK1 (dual-leucinezipper-bearing kinase 1), TAK1 (transforming growth factor β -activated kinase 1), TAO (thousand-and-one amino acid) 1 and 2, TPL2 (tumor progression loci 2), MLK3 (mixedlineage kinase 3), MEKK3 (MEK kinase 3) and MEKK4, and ZAK1 (leucine zipper and sterile- α motif kinase 1) [17]. The diversity of MAP3Ks and their regulatory mechanisms may provide the ability to respond to a wide range of stimuli and to integrate p38 MAPK activation with other signaling pathways. It should be noted that some MAP3Ks that trigger p38 MAPK activation can also activate the JNK pathway.

3.3. JNKs. The JNK pathway is known to be activated by cytokines, ligation of a variety of receptors, agents that interfere with DNA and protein synthesis, many other stresses, and to some extent by serum, growth factors, and transforming agents [18]. Two MEK family members, MEK4 (or MKK4) and MEK7 (or MKK7), have been implicated in phosphorylation of JNKs [18]. A number of different MAP3Ks can activate MKK4 and MKK7, suggesting that a wide range of stimuli can activate this MAPK pathway. These include MEKK1, 2, 3, and 4, MLK, and ASK1. In addition to its activation of MKK4 and MKK7, MEKK4 can also activate MKK3 or MKK6 to activate p38 MAPK pathway, which depends on the receptor activated and availability of other signaling molecules [18]. Research into the molecular mechanisms of oxidative stress-mediated activation of JNK and p38 pathways has focused on redox-sensitive proteins such as thioredoxin and glutaredoxin [19]. It is well known that ROS oxidizes thioredoxin to dissociate from ASK-1 for its activation, resulting in the activation of JNK and p38 pathways [20].

4. MAPK Phosphates

As above mentioned, MAPK pathways are activated through phosphorylation. Thus, the dephosphorylation of MAPKs by phosphatases is likely the most efficient mode of negative regulation. A number of protein phosphatases that are known to deactivate MAPKs include tyrosine, serine/threonine, and dual specificity phosphatases [21, 22]. A group of dual specificity protein phosphatases that are responsible primarily for dephosphorylation/deactivation of MAP kinases are often referred to as MAPK phosphatases (MKPs) [21, 22]. Since MKPs dephosphorylate MAPKs on their regulatory residues, aberrant regulation of MAPK activity may arise through defective regulation of the MKPs. The factors that activate MAPK pathways, such as environmental stresses and growth factor stimulation, can also activate MKP pathways [21, 22], supporting the notion that there is tight and specific control of MAPK activation and function by MKP activation. In mammalian cells, at least 11 MKP family members have been identified so far: MKP-1, MKP-2, MKP-3, MKP-4, MKP-5, MKP-7, MKP-X, PAC1, hVH3, hVH5, and MK-STYX. According to their subcellular localization, MKPs can be grouped: (1) MKP-1, MKP-2, hVH3, and PAC1 are found in the nucleus; (2) MKP-3, MKP-4, and MKP-X are found in the cytoplasm; (3) MKP-5, MKP-7, and hVH5 are found in both the nucleus and the cytoplasm [21, 22]. These MKPs exhibit distinct biochemical properties with regard to their substrate specificity [21, 22]. MKP-1 and MKP-2 show selectivity for p38s and JNKs over ERKs. MKP-3, MKP-X, and hVH3 primarily inactivate ERKs. MKP-5, MKP-7, and hVH5 show selectivity for JNKs and p38s, while MKP-4 and PAC1 inactivate ERKs and p38s.

MKP-1, the archetype, was initially discovered as a stress-responsive protein phosphatase [23]. Since MKP-1 deactivates MAPKs and is robustly induced by stress stimuli that also activate MAPKs, MKP-1 is regarded as an important feedback control mechanism that regulates the MAPKs. Compared with other MKPs, MKP-1 has been most closely examined. The activity of MKP-1 may be regulated at multiple levels, including transcriptional induction, protein stabilization, catalytic activation, and acetylation [24]. It has been reported that JNK and p38 pathways are highly activated in MKP-1-deficient mouse embryonic fibroblasts [25], supporting that MKP-1 functions as a critical negative regulator during MAPK activation. However, it should be noted that all MPKs may act cooperatively to modulate the MAPK pathways and to orchestrate appropriate cellular responses.

5. Activation of MAPK Pathways by ROS

Studies have demonstrated that ROS can induce or mediate the activation of the MAPK pathways [26]. A number of cellular stimuli that induce ROS production also in parallel can activate MAPK pathways in multiple cell types [4, 26]. The prevention of ROS accumulation by antioxidants blocks MAPK activation after cell stimulation with cellular stimuli [4, 26], indicating the involvement of ROS in activation of MAPK pathways. Moreover, direct exposure of cells to exogenous H_2O_2 , to mimic oxidative stress, leads to activation of MAPK pathways [27, 28]. The mechanism(s) by which ROS can activate the MAPK pathways, however, is not well defined. Because ROS can alter protein structure and function by modifying critical amino acid residues of proteins [5], the oxidative modification of signaling proteins by ROS may be one of the plausible mechanisms for the activation of MAPK pathways. However, the precise molecular target(s) of ROS is unknown.



FIGURE 2: Putative mechanisms for ROS-mediated activation of MAPK pathways. ROS are activated by growth factors, cytokines, and various stresses and rapidly removed by intracellular antioxidant proteins. ROS, once ROS production exceeds the capacity of the antioxidant proteins, may induce oxidative modification of MAPK signaling proteins (e.g., RTKs and MAP3Ks), thereby leading to MAPK activation. ROS may activate MAPK pathways *via* inhibition and/or degradation of MKPs.

Many growth factor and cytokine receptors have cysteine-rich motifs, the oxidation of which may activate MAPK pathways, and they, if not all, may be targets of oxidative stress. Meng et al. [29] determined the contribution of EGF receptor to activation of ERK pathway by insulinlike growth factor-I (IGF-I) in vascular smooth muscle cells. They showed that IGF-I induced phosphorylation of EGF receptor and ERK. AG1478, an EGF receptor inhibitor, inhibited IGF-I-induced phosphorylation of EGF receptor and ERK, suggesting that activation of ERK pathway results from EGF receptor activation. IGF-I stimulated ROS production and antioxidants inhibited IGF-I-induced ROS generation and activation of EGF receptor and ERK pathway, indicating that IGF-I activates ERK pathway through ROSmediated activation of EGF receptor. Moreover, Guyton et al. [30] investigated the factors controlling MAPK activation by the oxidant H_2O_2 . They found that H_2O_2 activates MAPK pathways via activation of growth factor receptors in several cell types.

ROS may also activate MAPK pathways through the oxidative modification of intracellular kinases (e.g., MAP3Ks) that are involved in MAPK signaling cascade. ASK-1, a member of the MAP3K superfamily for JNK and p38, binds to reduced thioredoxin in nonstressed cells. Upon an oxidative stress, thioredoxin becomes oxidized and disassociates from ASK-1, leading to activation of JNK and p38 pathways through oligomerization of ASK-1 [31]. A study has been shown that ASK-1-knockout mice exhibited lower levels of JNK and p38 activation in comparison to wild type after oxidant treatment [32]. Besides ASK-1, there may be other redox-sensitive MAP3Ks or MAP2Ks that can also activate MAPK pathways.

Another potential mechanism for MAPK activation by ROS may include the inactivation and degradation of the MKPs that maintain the pathway in an inactive state. Kamata et al. [33] demonstrated that intracellular H₂O₂ accumulation inactivates MKPs by oxidation of their catalytic cysteine, which leads to sustained activation of JNK pathway. Hou et al. [34] further confirmed that ROSinduced MKP inactivation causes sustained activation of JNK pathway. Choi et al. [35] showed that glutamateinduced oxidative stress induces sustained activation of ERK pathway through a mechanism that involves degradation of MKP-1. It is worth pointing out that ROS can upregulate MKP-1 expression. Zhou et al. [36] found that upregulation of MKP-1 expression by H₂O₂ correlates with inactivation of JNK and p38 activity. Kuwano and Gorospe [37] revealed that the oxidant-triggered induction of MKP-1 is potently influenced by two posttranscriptional processes, mRNA stabilization and increased translation. Lornejad-Schäfer et al. [38] investigated the regulation of MKP-1 expression and JNK activation by the induction of light damage that has shown to enhance ROS production in ARPE-19 cells. In their study, low light doses upregulated MKP-1 expression in ARPE-19 cells, this being accompanied by inactivation of JNK pathway. High light doses, however, led to a decrease in the expression of MKP-1, resulting in sustained activation of JNK pathway. Hence, the paradox in the roles of ROS as "inducers" in the regulation of MKP-1 expression and as "inhibitors" may be, at least in part, related to differences in the concentrations of ROS.

6. Conclusion

The evidence supporting that ROS can activate MAPK pathways at cellular levels is based largely on the following findings: (1) cellular stimuli that are capable of producing ROS can also activate MAPK pathways in a number of different cell types, (2) antioxidants and inhibitors of ROSproducing enzymatic systems block MAPK activation, and (3) exogenous addition of H₂O₂, one of ROS, activates MAPK pathways. The putative mechanisms by which ROS, on the basis of their oxidation potentials, can activate MAPK pathways may include (1) oxidative modifications of MAPK signaling proteins (e.g., RTKs and MAP3Ks) and (2) inactivation of MKPs, as illustrated in Figure 2. Finally, the site of ROS production and the concentration and kinetics of ROS production as well as cellular antioxidant pools and redox state are most likely to be important factors in determining the effects of ROS on activation of MAPK pathways.

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Review Article The Dynamical Systems Properties of the HOG Signaling Cascade

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The High Osmolarity Glycerol (HOG) MAP kinase pathway in the budding yeast *Saccharomyces cerevisiae* is one of the best characterized model signaling pathways. The pathway processes external signals of increased osmolarity into appropriate physiological responses within the yeast cell. Recent advances in microfluidic technology coupled with quantitative modeling, and techniques from reverse systems engineering have allowed yet further insight into this already well-understood pathway. These new techniques are essential for understanding the dynamical processes at play when cells process external stimuli into biological responses. They are widely applicable to other signaling pathways of interest. Here, we review the recent advances brought by these approaches in the context of understanding the dynamics of the HOG pathway signaling.

1. Introduction

Living organisms have evolved specialized biochemical pathways to cope with stressful, often changing environments. Even in simple cells such as yeast, thousands of specialized sets of sensing and signaling proteins form modules used to monitor and adapt to the environmental state and its variations. Such modules can be insulated or, on the contrary, connected to one another. Whereas insulation allows for robust and sensitive response, the interconnection of modules allows for higher-level behavior such as multiple input sensing and decision making through cross-talk [1]. For a given stimulus, the biochemical components of the different modules that play a role in the cellular response are usually well described in the literature. Their biological functions and interactions are known in detail, especially in model organisms such as the budding yeast. This knowledge comes from decades of complex, tedious, and elegant experiments. Genetic techniques such as gene deletion, mutation, and overexpression have been used to infer the connection patterns between proteins and the architectures of many modular functions.

Biochemical assays provided crucial information on protein phosphorylation and kinase activity. Microarrays revealed the role of these modules in determining global gene expression.

Signaling pathways are naturally dynamic [2] in that cells must respond to external signals in a timely manner, and indeed, the cellular response is often affected by the temporal properties of the external signal. In addition, the internal dynamics and timing of events in the signaling pathway determine the cellular response. These internal dynamics determine the information flow, allowing cells to process and convey information from a sensory input to a specific protein in charge of orchestrating the cellular response [3]. Until recently, experimental techniques have been limited such that most studies have examined the response of a signaling pathway to a stationary stimulus. Accordingly, adaptation and cellular responses to environmental cues were usually studied only with respect to the magnitude of the stimulus without seriously taking into account dynamical aspects. Identification of the components of a signaling pathway through the techniques mentioned above, combined with studies of simple stationary stimuli, is not enough to understand the dynamics or systems-level properties of a complex biological network.

With the emergence of systems biology, there has been an important paradigm shift, and it is becoming increasingly clear that the temporal variations of stimulatory inputs can be directly sensed by cells [5] and that studying cells in time-variable environments is a powerful way to determine signaling pathway architecture and to understand how they process information [6, 7]. Experimental microfluidicsbased strategies have matured to allow for excellent control of the cellular environment both in time and space [8, 9]. This technology coupled with genetic engineering to fluorescently tagged proteins allows for real-time observation of the system's response using fluorescence microscopy. Finally, quantitative real-time measurements form the basis for the development of mathematical models and the use of signal analysis tools, such as reverse engineering, to model the dynamical aspects of signaling pathways [10]. These models in turn provide testable experimental predictions.

This review describes the recent strategies that have been developed to assess quantitatively the dynamics of the canonical HOG MAP kinase (MAPK) pathway in the yeast, *Saccharomyces cerevisiae*. We shall first briefly review the key characteristics of the organization of the HOG pathway. We then discuss the novel experimental and modeling tools [10, 11, 16–18] that are allowing new insights into the pathway's dynamics and systems-level behavior.

2. MAPK Cascades in Yeast

Among signaling pathways, the Mitogen Activated Protein Kinases (MAPK) family has received considerable attention. MAPK pathways are very well conserved from yeasts to mammals [19-21] and several comprehensive reviews are available in the recent literature [22, 23]. MAP Kinase pathways are involved in many cellular processes such as stress response, the regulation of differentiation and proliferation. These pathways contain a canonical module of three protein kinases that act in series (Figure 1). Upon phosphorylation by an upstream protein, a MAP kinase kinase kinase (MAPKKK) phosphorylates a MAP kinase kinase (MAPKK) on conserved serine and threonine residues, which in turn phosphorylates a MAP kinase (MAPK) on a threonine (sometimes serine) and a tyrosine residue located adjacent to each other and separated by a single amino acid (Thr/Ser-X-Tyr). This dual phosphorylation site is located in the activation loop of the catalytic domain and its dual phosphorylation is needed for activation of the MAP kinase.

There are five MAPK modules in yeast (Table 1) [22]. The hyperosmotic glycerol (HOG) pathway is activated in response to a hyperosmotic stress [24–28]. The Cell Wall Integrity (CWI) module controls the cell wall integrity and is triggered in response to numerous stresses including cell wall deterioration, temperature shifts, and hypo osmotic shocks [29–31]. The pheromone pathway [32, 33] controls the mating response which involves an important morphological deformation of yeast cells. Finally, the filamentous growth pathway [33, 34] and the sporulation pathway [22] control



FIGURE 1: The canonical structure of a MAPK cascade. We used the Systems Biology Graphical Notation (SBGN) [4] to represent the interactions between the MAP Kinases. Activations of MAPK occur through enzymatic phosphorylation and ATP consumption. Interactions with other components and in particular with phosphatases are not shown. In the case of the HOG pathway in yeast, dual phosphorylation of the final MAPK (Hog1p) occurs within a few minutes after an hyper-osmotic stimulus.

the response to starvation for haploid and diploid cells although the sporulation pathway is not as well known as the other four MAPK pathways. Only its MAPK has been identified in diploid cells (Smk1p), and it is thought to drive the spore cell wall assembly [22]. Though they share numerous components, the five MAPK pathways of the yeast *Saccharomyces cerevisiae* are tightly regulated by crosstalk and mutual inhibition which permit faithful signaling, adaptation to their environment, and regulation of growth and morphogenesis [22]. Among these MAPK pathways, the HOG pathway (Figure 2) is particularly well suited to study signaling dynamics, since it can be reliably activated through increasing the osmolarity of the environment.

3. The HOG MAPK Signaling Pathway

Water homeostasis is fundamental for life. In nature, the environment can vary rapidly from isotonic to hyper or hypo osmotic conditions, and yeast cells have to adapt quickly [23, 47]. The first response after a hyperosmotic shock is the rapid loss in few seconds of cell volume due to water efflux and the activation of membrane sensory receptors followed by the activation of the HOG pathway which is completed after a few minutes (Figure 2) [11, 48]. Two distinct branches of the pathway detect changes in osmolarity and activate the

TABLE 1: The MAPK pathways in *S. cerevisiae*. The morphological adaptation corresponds to the cell behavior in response to each specific signaling input. The major molecular actors for each pathway are indicated below. Spore cell wall assembly during sporulation is another morphogenetic process driven by a MAPK protein (Smk1p), but with little knowledge on the other proteins involved and the structure of the pathway.

External Stress	Pheromone	Starvation	Hyperosmolarity	Cell wall Stress
Morphological Adaptation	0 0	000		0
Membrane Sensors	Ste2/3	Sho1	Sln1, Sho1, Msb2, Hkr1, Opy2	Wsc1, Mid2
MAPKKK	Stel1	Ste11	Ssk2/22 & Ste11	Bck1
MAPKK	Ste7	Ste7	Pbs2	Mkk1/Mkk2
MAPK	Fus3/Kss1	Kss1	Hog1	Slt2
Transcription factors	Ste12 [22, 35]	Ste12, Tec1 [22, 35, 36]	Hot1, Sko1, Smp1, Msn2/4 [22, 28, 37, 38]	Rlm1, Swi4/6 [22, 39, 40]
Inhibition	Msg5, Ptp2, Ptp3 [21, 41, 42]	[21]	Ptcs, Ptp2, Ptp3 [21, 43–45]	Msg5, Ptp2, Ptp3, Sdp1 [21, 30, 46]

pathway. These branches converge at the level of the MAPKK Pbs2p. The first branch is referred to as the SHO1 branch [23, 49], while the second is referred to as the SLN1 branch [50, 51].

Sln1p negatively regulates the HOG signaling pathway and deletion of SLN1 is lethal due to pathway overactivation. This lethality is suppressed by knocking out any of the downstream components SSK1, SSK2/SSK22, PBS2, or HOG1. Sln1p contains two transmembrane domains, a histidine kinase domain and a receiver sequence. Sln1p autophosphorylates on its histidine kinase domain. The phosphate group is then transferred to its receiver domain, then to Ypd1p and finally to Ssk1p. This set of three proteins forms a phosphorelay [50, 51], a very common signaling motif in prokaryotes [51], but rare in eukaryotic cells such as yeast. The phosphorylated form of Ssk1p is inactive and the downstream MAPK pathway is usually not activated. However, after a hyperosmotic stress, Sln1p is inactivated by an unknown mechanism (though it has been proposed that Sln1p is sensitive to membrane tension [23, 52]) leading to the inactivation of Ypd1p and derepression of Ssk1p. Finally, unphosphorylated Ssk1p binds to the MAPKKKs Ssk2p and Ssk22p, which autophosphorylate, and then can phosphorylate the MAPKK Pbs2p. Sln1p seems to dominate the kinetic response of the pathway while also ensuring its robustness by inducing high basal Hog1p expression counteracted by a fast-acting negative feedback to allow rapid pathway response [53]. Thus, this tightly tuned signaling branch allows rapid and sensitive responses to environmental changes.

Sho1p consists of four transmembrane domains and an SH3 domain. This domain permits the recruitment of molecular actors, notably the MAPKK Pbs2p, to the plasma membrane [54]. The upstream kinase Ste20p, the G-protein Cdc42p, and the MAPKKK Ste11p needed for the activation of the protein Pbs2p are also recruited to the membrane [55]. Since it is a transmembrane protein, Sho1p has long been considered an osmosensor [56]. However, recent studies suggest that Sho1p is more an anchor protein than a sensor for osmolarity [55]. Hkr1p and Msb2p, two mucin-like [57– 60] proteins that form heterooligomeric complexes with Sho1p [58, 59] have recently been proposed as osmosensors of the SHO1 branch. Components of the SHO1 branch also take part in pseudohyphal development and mating, indicating that Sho1p might not have a specific role in osmosensing but a more general role related to cell shape measurement [61].

MAPKKKs of these two initiating branches induce the phosphorylation of the MAP kinase kinase Pbs2p on the conserved residues Ser514 and Thr518 [62]. Pbs2p is a cytoplasmic protein essential for the activation of Hog1p by dual phosphorylation on the conserved Thr174 and Tyr176 [62]. PBS2 and HOG1 are essential for osmoadaptation as null mutations in both genes induce osmosensitivity [23, 63]. Pbs2p also plays the role of a scaffold for the SHO1 branch [49, 54, 56, 64] by anchoring the different components, promoting signal propagation between proper protein partners and preventing improper cross-talk between the Pheromone pathway and the HOG pathway. Once Pbs2p phosphorylates Hog1p, Hog1PP translocates to the nucleus in a manner that is dependent upon the karyopherin Nmd5p [65]. Localization of Hog1p-GFP to the nucleus can be used as a reliable reporter of pathway activity.

4. Sequential Response after a Hyperosmotic Shock

The activation of the Hog1p MAPK triggers several responses on different time-scales (Figure 3) [48]. A rapid nontranscriptional response in the cytoplasm corresponds to the closure of Fps1p [66] and the activation of several kinases (e.g., Rck2p [67], Pfk2p [68]). Fps1p belongs to the ubiquitous Major Intrinsic Protein (MIP) [69] family and is known to play a central role in yeast osmoadaptation by controlling both uptake and efflux of the osmolyte



FIGURE 2: The HOG pathway. View of the main molecular actors involved in the hyperosmotic glycerol pathway (see text for more details). Two branches led by Sho1p and Sln1p are sensitive to high osmolarity and lead to the activation of Pbs2p and Hog1p after a hyperosmotic shock. Hog1p has both a cytoplasmic and a nuclear role, with different timescales, that correspond to a fast non transcriptional response and a longer response involving transcription when dealing with strong hyper osmotic shock. The yeast pictures at the bottom show nuclear localization of Hog1p tagged by GFP after a moderate hyper-osmotic shock (Sorbitol, 1 M). Colocalization with the nucleus is seen on the overlay pictures between the GFP channel (Hog1p) and the RFP channel (Htb2p). Note that localization is transient and reversible if the cell is put back into isotonic conditions.

glycerol [70]. Importantly, Fps1p is gated by osmotic changes [66, 71]. Indeed, this channel protein is closed under hyperosmotic stress to enable intracellular accumulation of glycerol, whereas it is open under low-osmolarity conditions to allow for glycerol efflux.

On a longer time scale, several minutes after an osmotic shock, Hog1p induces the modification of expression of nearly 600 genes [72–75]. This transcriptional response is driven by intermediate transcriptional factors: Hot1p, Sko1p, Smp1p, and Msn2/4p [37, 38, 74, 76, 77]. Importantly, Hog1p initiates glycerol biosynthesis via the transcriptional factor Hot1p [38]. Glycerol production is due to the expression of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. Both enzymes are encoded by two similar isogenes, *GPD1*, *GPD2* and *GPP1*, *GPP2*, respectively, [78, 79]. The accumulation of glycerol results in an increase of the internal osmolarity, leading to water influx and cell size recovery. Hot1p is also involved in regulating glycerol influx by inducing a strong and transient expression of STL1, which codes for a glycerol proton symporter located in the plasma membrane [80]. Hog1p is dephosphorylated and exported from the nucleus via the karyopherin Xpo1p [65] 20 to 30 minutes after an osmotic shock depending on the severity of the shock. This is concomitant with the onset of glycerol production and restoration of osmotic balance. Dephosphorylation of Hog1p is due to nuclear phosphatases. Phosphatases have a critical role in downregulation of MAPK proteins whose excessive activation can be lethal for the cell. In yeast, three classes of protein phosphatases are known to downregulate MAPK pathways. The dual specificity phosphatases (DSPs) dephosphorylate both phosphotyrosine (pY) and phosphothreonine (pT). The protein tyrosine phosphatases (PTPs) dephosphorylate only tyrosine residues. Finally, protein phosphatases type 2C (PTC) dephosphorylate threonine, serine, and sometimes tyrosine residues. For the HOG pathway, the serinethreonine phosphatases Ptc1p, Ptc2p, and Ptc3p act on both the Pbs2p (MAPKK) and Hog1p (MAPK), while the tyrosine phosphatases, Ptp2p and Ptp3p strictly control Hog1p [43, 44, 49]. Ptp2p is predominantly localized in the nucleus, Ptp3p in the cytoplasm, while the protein phosphatases types 2C are located both in the cytoplasm and in the nucleus. Simultaneous knockout of both PTP2 and PTC1 is lethal for the cell [45]. Deletion of PTP3 induces overactivation of Hog1p but is not lethal because it predominantly acts on other MAPK proteins involved in the mating pathway.

5. Towards a Model of the HOG Pathway

Years of genetic and biochemical analysis have provided us with an extraordinarily precise description of the key players in the HOG pathway. What about the signaling dynamics of the pathway? How does the architecture determine the pathway's signal processing ability? Classic molecular biology experiments were based on step shock experiments with an osmotic agent, such as NaCl or sorbitol at various concentrations. Phosphorylation states of key proteins have been measured at different time points after a step shock at the population level, showing a transient increase of phosphorylation (lasting several minutes) concomitant with nuclear enrichment of Hog1p [81]. Nuclear cytoplasmic shuttling of Hog1p was also observed qualitatively, indicating a fast deactivation of the pathway when cells are returned to an isotonic environment [81]. Levels of gene expression have been measured at different timepoints after an osmotic shock using microarrays [73]. Although done with a low resolution in time compared to biophysical experiments, these measurements give an idea of the dynamics of the activation of the pathway.

Based on such measurements, several models have been proposed to describe mathematically the HOG signaling pathway and more generally osmoadaptation in yeast [82]. The most comprehensive and the first integrative one is due to Klipp et al. [81]. Their model takes not only the HOG signaling cascade into account (only the SLN1 branch), but



FIGURE 3: Sequential sketch of yeast adaptation to a hyperosmotic shock. The evolution with time of the size, phosphorylation of Hog1p, and internal concentration of glycerol are schematically represented in the center of the picture. (1) After an increase of the external osmolarity (green), a first mechanical response corresponds to a rapid loss of water (blue arrow). It leads to a decrease of the cell size and a loss of turgor pressure. (2) HOG osmosensors (blue) activate the pathway and eventually lead to the phosphorylation of Hog1p. (3) Hog1PP induces several processes: (a) Inactivation of the glycerol channel Fps1p preventing glycerol leakage; (b) direct or indirect activation of cytoplasmic actors, for example, 6-phosphofructo-2-kinase (Pfk2p) involved in glycerol synthesis; (c) translocation in the nucleus. Note that there are other targets of Hog1p such as Sic1p, Hs11p, Nha1p, and Tok1p. (4) Nuclear Hog1PP induces a large transcriptional response. In particular, the gene GPD1 leading to glycerol synthesis is upregulated. Negative feedbacks (glycerol production, phosphorylation of Sho1p, etc.) allow inhibition of the pathway activity. (5) Increase of the internal glycerol leads to water influx and progressive cell size recovery while Hog1p is exported from the nucleus. (6) Pathway is off, and turgor pressure and cell size are restored. The cell is adapted to its new environment.

also includes a description for the metabolic production of glycerol, as well as an elementary gene expression model for the enzymes involved in glycerol production. The model also includes the closure of the membrane glycerol tranporter Fps1p and takes the dephosphorylation of nuclear Hog1p by Ptp2p into account. Most reactions in the model were described by the mass action rate law. The model consisted of 70 parameters, of which 24 had to be estimated. To estimate this number of parameters with the limited data available, the authors divided the model in modules and fitted them separately to data points. Their model reproduced accurately the transient response of the HOG pathway after a single hyperosmotic shock. This included the phosphorylation states of Hog1p and Pbs2p, as well as glycerol production and cell-size recovery. In addition, the model was able to correctly predict the effect of different mutations of proteins

involved in the pathway. Mutants unable to produce glycerol $(gpd1\Delta, gpd2\Delta)$ [83] or to close the Fps1p channel showed an increased duration of HOG activity. Mutants with an increased phosphatase Ptp2p activity showed a lower level of phosphorylated Hog1p but a similar period of HOG activity.

Although very promising, such an approach is still extremely difficult to fine tune since it relies on many unknown parameters. Comparison of the model outputs to experimental data is crucial. To further constrain and test complex models one needs quantitative, time-resolved experiments at the single-cell level in response to complex input signals.

As engineers do with electronic circuits and chips, a very powerful way to explore the dynamics of a given system is to observe its response to complex input signals. Such an approach lends itself to developing minimal models that



FIGURE 4: Different microfluidics techniques to control the chemical environment of single yeast cells while imaging them through microscopy. (a) Microfluidic system as described in Hersen et al. [11]. Yeast cells are fixed in the channel by the lectin protein Concanavalin A. One inlet is filled with an iso-osmotic media (blue) and the other with a hyperosmotic media (orange). By tightly controlling the pressure in each inlet, it is possible to create a periodic shock on the cells. (b) Optical tweezers system (red) as described by Eriksson et al. permits to control the cells position in the channel with two fluids flowing side by side [12]. (c) The system developed by Charvin et al. uses a dialysis membrane (green) to trap cells on top of a soft PDMS slice [13]. (d) Multilayer microfluidic device [14]. The top layer (green) is used to capture cells. By controlling the pressure inside this channel, cells can be optimally trapped while subjected to periodic shocks. The bottom layer is used to culture cells.

capture the dynamical properties of the pathway, such as feedback loops and signal processing abilities, without taking into account all the details of the biochemical reactions. These approaches require designing experimental systems in which the extracellular environment can be quickly and precisely varied. We will now review the innovative methodologies that have been recently used to study single yeast cells in time varying environments. Then, we will review how those measurements have been integrated into minimal modeling to further study the dynamics of the HOG pathway.

6. Fast Control of the Chemical Environment of Single Cells

Several approaches, using microfluidics [8, 9, 84, 85], have been recently proposed to allow for a fast and reliable control of the chemical environment of yeast cells [7]. Hersen et al. [11] designed a fast binary switch to repeatedly change the environment of single yeast cells between two chemical conditions as fast as every second (Figure 4(a)). They used a Y-shaped flow chamber, 50 μ m high and 500 μ m wide, with two inlets. One inlet was filled with an isotonic medium, and the other with the same culture medium complemented with sorbitol to increase its osmolarity. At such small scales, flows are laminar and fluids do not mix but rather simply flow side by side. The lateral position of the fluids interface is set by the relative hydrostatic pressure-or the relative flux-of the two inlets. Changing this pressure difference displaces the interface laterally in less than a second. Yeast cells, previously fixed in the channel through concanavalin-A coating were then repeatedly switched from an isotonic to a hyperosmotic environment. An interesting alternative developed by Eriksson et al. [12] consists of moving the cells with optical tweezers (Figure 4(b)) rather than moving fluids over fixed cells. This strategy removes the potential influence of cell adhesion on signaling dynamics related to morphological changes, but at the cost of technological complexity. Also, such a strategy is very time consuming. Holographic tweezers-a sophisticated version of optical tweezers-can help to increase the number of cells that can be observed in real time [86]. Another strategy was proposed by Charvin et al. [13, 87]. Yeast cells are fixed between a permeable dialysis membrane and a cover slip coated with a very thin layer of soft PDMS (Poly-Di-MethylSiloxane). A channel is placed on top of the membrane and allows flow of fresh media and exchange within a few minutes. Nutrients and other chemicals can freely diffuse through the membrane. With this device, environmental exchange happens more slowly, but cells can grow over several generations in a monolayer simplifying their observation through microscopy. Indeed, Charvin et al. used it to force periodic expression of cyclins in yeast growing exponentially up to 8-10 generations.

More complex devices have been proposed, though they require a high degree of expertise to fabricate and manipulate. Bennet et al. [88] developed an environmental switcher capable of generating sinusoidal inputs. Their multilayer device was composed of a microchemostat, with a depth of $4\,\mu\text{m}$ to force yeast cells to grow in a monolayer, and a fluid mixer to generate complex time varying environmental signals for the cells in the chemostat chamber. They used this device, in a particularly elegant work, to revisit the wiring of the GAL system in yeast, by subjecting cells to sinusoidal inputs of carbon source over a range of frequencies. Taylor et al. [14] described a high throughput microfluidics single-cell imaging platform to study the dynamics of the pheromone response in yeast. They combined a fluidic multiplexer, an array of channels, and many sieve valves to trap cells and to control fluid delivery. They were able to perform simultaneous time lapse imaging of 256 chambers with 8 different genotypes with several dynamical inputs. Such a strategy, although very sophisticated, can enhance dramatically the quantity of data gathered to improve our knowledge and refine modeling of MAPK pathways in yeast [7].



FIGURE 5: Schematic representation of the Hog pathway models of Mettetal et al. [10] and Zi et al. [15]. Pictures are redrawn from original figures of these papers. Top: (a) Diagrammatic representation of Mettetal's model. Au(t) represents the osmolarity applied at time *t* and the variables *x* and *y* can be identified with the intracellular glycerol concentration and the enrichment of Hog1 in the nucleus. The model contains a feedback depending on Hog1p (with strength β) and one, which is independent of Hog1p (strength α). The equations for this model read $\dot{y} = (A_0u - x) - \gamma y$ and $\dot{x} = \alpha(A_0u - x) + \beta y$. (b) The same model, interpreted in biological terms. The export of osmolytes is regulated by a mechanism, which does not depend on the MAPK pathway (e.g., closure of Fps1p) and by a mechanism depending on Hog1p (a slow transcriptional and a fast nontranscriptional). Both of these feedbacks act by increasing the production of glycerol.

7. New Insights from Coupling Complex Stimulus and Reverse Systems Engineering

Using such microfluidics strategies (Figure 4(a)), Hersen et al. studied the HOG pathway response to periodical osmotic stimulation over a range of frequencies. Interestingly, the HOG pathway acts as a low-pass filter, meaning that the output of the pathway (Hog1p nuclear localization) does not follow a fast varying input precisely, but rather integrates fast fluctuations over time. For wild-type strains, when the input signal varies slower than once every 200 s, Hog1p cytoplasmic—nuclear shuttling follows the input variations faithfully [11, 17]. However, when the input varies more rapidly than every 200s, Hog1p nuclear translocation no longer follows the input faithfully, but instead integrates over the input fluctuations [11, 17]. This typical time is also the slowest time (or limiting step) of activation of the pathway although it was not possible from these experiments to point out which biochemical step was limiting. By genetic removal of one of the two branches, the contribution of each branch was also measured by Hersen et al., and it was found that the SHO1 branch is slower than the SLN1 branch by almost a factor two. The SHO1 branch was actually unable to integrate the too fast variations of the input whereas the SLN1 branch, when taken alone, was displaying a similar behavior than wild-type cells [11]. Those investigations clearly evidenced that the pathway can be turned off very quickly and repeatedly, suggesting the existence of several feedback loops acting on different timescales.

An attempt to decipher the dynamical aspects of these feedback loops has been done by Mettetal et al. [10], who also examined the response of the Hog1p nuclear localization in response to an oscillating input. They constructed, based on these frequency experiments, a simple predictive model, which was not based on biological knowledge (Figure 5(a)). Subsequently, they identified the two variables of their model with the intercellular osmolyte concentration and the phosphorylation state of Hog1p and concluded that the pathway contains a Hog1-dependent and a Hog1-independent feedback mechanism. By underexpressing Pbs2p, thereby reducing the sensitivity of the Hog1-response to the input, they were able to isolate the Hog1-independent feedback from the Hog1-dependent feedback. Based on this they concluded that the Hog1-dependent feedback is required for fast pathway inactivation. By inhibiting translation, they showed indeed that the slow transcriptional response triggered by Hog1p is only necessary for the adaptation to multiple osmotic shocks, while for a single osmotic shock faster nontranscriptional feedback mechanisms dominate the response. Their conclusion is in perfect agreement with recent experimental investigations showing that even cells with Hog1p anchored to the membrane present an increase of glycerol production after a hyperosmotic shock [89]. Although the details are not known, Hog1p directly or indirectly activates the 6-Phosphofructo-2-kinase (PFK2) [68] which leads to an increase production of glycerol through Gpd1p activity.

Hao et al. also focused on rapid non-transcriptional feedback loops. First, they noticed that the response of the SHO1 branch is more transient than that of the SLN1 branch. Then, based on previous observations, they constructed three simple mathematical models, each describing another possible mechanism of HOG inactivation. One model was based on Hog1p mediating activation of a negative regulator (phosphatases), while the other two models focused on the negative control of a positive regulator. Analysis of the different models suggested a Hog1p-dependent feedback mechanism occurring early in the response. Their experimental analysis confirmed this and suggested that Hog1p acts negatively on Sho1p by phosphorylation, thereby implementing a direct negative feedback loop.

Muzzey et al. [18] followed a similar approach to study the feedback mechanisms within the pathway. They identified the transient activation of Hog1p with a feature called perfect adaptation, which states that the steady state output of the pathway does not depend on the strength of the osmotic shock. They argued that robust perfect adaptation requires at least one negative feedback loop containing an integrating component [90] and they analyzed the location of this integrator. They defined an integrating component as a dynamic variable whose rate of change does not depend on itself. They monitored multiple system quantities (cell volume, Hog1p, and glycerol) and used varied input waveforms to analyze the pathway. Similar to Hao et al. [16], they constructed different variants of a mathematical model, each with a different location of the integrating component. The authors found that the integral feedback property is Hog1p dependent and regulates glycerol uptake.

More recently, Zi et al. [15] analyzed the experimental frequency response of the HOG pathway done by Hersen et al. and Mettetal et al. They constructed a minimal model that can reproduce the response of the pathway to oscillating inputs (Figure 5(b)) [15]. They defined a signal response gain, which is defined as the ratio of the integrated change of the output of the pathway to the integrated input change and represents a measurement for the efficiency of signal transduction. They concluded that yeast cells have optimized this signal response gain with respect to certain durations and frequencies of osmotic variations.

These different analyses have shown that the HOG signaling cascade can be described in a very simple and modular way with several feedback loops operating to deactivate the pathway: two operating on short time scales through Hog1p activity (Sho1p deactivation and glycerol production increase), and one depending on transcriptional activation of GPD1. The dynamics of the pathway was also precisely measured and it was shown that it behaves as a low-pass filter with a cutoff frequency, probably set by protein concentration. Interestingly, the SHO1 branch which is known to be involved in other cellular processes was shown to be slower in activating the Hog1p MAPK than the SLN1 branch. Finally, those approaches have provided us with an easily tractable mathematical model of the HOG pathway that can be efficiently coupled to detailed mechanistic models to study in silico the behavior of this MAPK pathway. Taken together, the coupling between mathematical modeling and experimental frequency analysis of the HOG pathway has given very important insights into the HOG pathway dynamics and more generally its functioning, demonstrating the interest of developing such strategies for studying signaling pathways in yeast.

8. Future Directions

Although the structure and the dynamics of the HOG signaling pathway are now well understood, several key points remain to be elucidated, the most elusive one being the mechanistic functioning of the two osmosensors, Sln1p and the Sho1p complex. Another important aspect of a better understanding of the HOG pathway is to integrate its behavior with other cellular processes. In particular, in 2000, Gasch et al. [73] compiled genome expression profiles of *S. cerevisiae* yeast subjected to several stress conditions and discovered that genes normally induced after a hyperosmotic shock are downregulated in response to a hypo-osmotic shock and vice versa. The CWI pathway is activated by hypo-osmotic stimulation [29], its physiological role being to reinforce the cell wall and prevent the cell from bursting. HOG and CWI do not share direct components but were

seen to interact with each other [91, 92]. During cell growth both pathways may well be activated and deactivated within short intervals to balance between cell expansion and cell wall development. The Sln1p-dependent response regulator Skn7p [93, 94] could have a role in linking the cell-integrity pathway to the HOG pathway. Skn7p also interacts with Rho1p an upstream component of the CWI pathway. The evidence that Skn7p is apparently controlled by sensors of both the HOG pathway and the cell-integrity pathway makes Skn7p an excellent candidate for a regulator that coordinates osmoregulation and cell wall biogenesis [23, 93, 94]. More work is needed to better understand the putative role of Skn7p in coordinating different aspects of turgor pressure control and cell surface assembly. Using minimal models and fluctuating environments to activate periodically the CWI and/or the HOG pathway is one interesting way to explore their interactions. Similarly, it is known that the HOG pathway and the Pheromone pathway can interact [95–98]. For example, a $hog1\Delta$ strain will respond to a hyperosmotic shock by activating the response to pheromone pathway. Again, the dynamics of such cross-talk has not been intensely studied. Performing time varying inputs with both pheromone and hyperosmotic medium will provide invaluable experimental data to probe for the dynamical aspects of cross-talk between MAPK in yeast.

Since MAPKs pathways are highly conserved from yeast to mammalian cells, it would be interesting to test higher eukaryotic cells, in single cell experiments, for similar system level properties. Although more difficult to implement than for yeast cells, microfluidic technics can also be used to control the external environments of mammalian cells both in time and space. Transposing the approaches described here to mammalian cells will probably give further insights in their signaling pathways dynamics.

9. Conclusion

Since its initial discovery in 1993 [24], extensive molecular and genetic research has uncovered the molecular actors, interactions, and functions of the components in the HOG signaling pathway. However, these methods are limited in that one cannot predict the behavior of a complex system from the analysis of isolated components. Understanding of the entire system requires the use of novel techniques borrowed from engineering, physics, and mathematics. Microfluidic technologies combined with livecell microscopy have allowed the use of temporally complex stimuli to interrogate pathway function. Kinetic information obtained through biochemistry combined with knowledge of the molecular components has allowed for complex quantitative models of the HOG pathway to be constructed. These models in turn provide experimentally testable predictions about pathway behavior and function. Simple "blackbox" models designed to mimic only key components of the pathway have proven useful for understanding specific phenomena. Thus, genetic and biochemical data combined with novel experimental approaches and modeling have allowed for the prediction of the dynamics and systems-level

properties of HOG pathway signaling processes. These techniques are easily extended to other signaling pathways of interests with the final goal being to understand the relationships between structure, kinetics, and dynamics at the systems-level in complex biological networks.

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Review Article

Parasite Mitogen-Activated Protein Kinases as Drug Discovery Targets to Treat Human Protozoan Pathogens

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Protozoan pathogens are a highly diverse group of unicellular organisms, several of which are significant human pathogens. One group of protozoan pathogens includes obligate intracellular parasites such as agents of malaria, leishmaniasis, babesiosis, and toxoplasmosis. The other group includes extracellular pathogens such as agents of giardiasis and amebiasis. An unfortunate unifying theme for most human protozoan pathogens is that highly effective treatments for them are generally lacking. We will review targeting protozoan mitogen-activated protein kinases (MAPKs) as a novel drug discovery approach towards developing better therapies, focusing on *Plasmodia, Leishmania*, and *Toxoplasma*, about which the most is known.

1. General Properties of MAPKs

Virtually all eukaryotic organisms possess MAPKs, signal transduction molecules that regulate cell functions such as tissue morphogenesis, cytoskeletal rearrangements, proliferation, differentiation, survival, immune responses, and adaptation/stress-response [1-3]. Encephalitozoon cuniculi is the only example to date of a eukaryote apparently lacking any MAPKs [4]. The MAPK superfamily, which evolved 1.0 to 1.5 billion years ago [5], comprises proline-directed serine/threonine kinases that are classified based on the primary amino acid sequence within the catalytic domains and must possess a [TS]XX[LIVM]XT[RK] [WY]YRXPEX[LIVM] signature sequence at its core [6-8]. The phosphorylation lip (solid underline beneath the sequence above) is required for MAPK activation by upstream regulators and is contiguous with the proline-directed (P+1) peptide binding pocket (double underline beneath the sequence above), conferring substrate specificity, and is capable of being singly [(pT)XX)] or dually [(pT)X(pY)] phosphorylated in response to particular extracellular stimuli [9]. In addition, MAPKs possess 11 subdomains [5, 10] with numerous highly conserved residues required for ATP binding, phosphotrans-ferase activity, and substrate specificity [7].

MAPKs are often controlled by highly evolutionarily conserved regulatory cascades involving sequential phosphorylation by three component modules consisting of MAPK kinase kinases (MKKKs, Ste11-like kinases) and MAPK kinases (MKKs, Ste7-like kinases), terminating in the phosphorylation of specific MAPKs [11]. Many MAPK cascades have recently been expanded to include a fourth tier involving proteins aptly termed MKKKKs (Ste20-like kinases) [12] that can either serve in a noncatalytic capacity as a scaffold to promote pathway assembly (and MKKK autoactivation) or can phosphorylate specific MKKKs [13]. Once activated, MAPKs phosphorylate a wide variety of proteins including MAPK-activated protein kinases and transcription factors, ultimately resulting in changes in gene expression [14, 15]. MAPK signaling can also have additional epigenetic effects by affecting histone modification [16].

MAPKs are grouped into subfamilies on the basis of amino acid sequence similarity, mechanism of activation,

and the type of MAPK cascade to which they belong. Cyclindependent kinases share very high amino acid sequence identity with MAPKs [17] but generally lack a phosphorylation lip. Differences in the precise amino acid composition of the phosphorylation lip have historically been used to classify MAPKs, as outlined below. Our phylogenetic studies [18] have established, however, that homology between many short strings of amino acids found in MAPKs is of equal or greater importance when classifying MAPKs within different subfamilies.

Four conventional MAPK subfamilies exist, which are also described as "typical", that is, capable of dual phosphorylation [8]. These conventional MAPK groups include the extracellular signal-regulated kinases (e.g., mammalian ERK1 and ERK2, possessing a TEY motif at the phosphorylation lip, [19]), c-Jun-activated kinases (e.g., mammalian JNK1, JNK2, and JNK3 (TPY motif) [20]), p38 stressresponse MAPKs (e.g., mammalian $p38\alpha$, $p38\beta$, $p38\gamma$, and $p38\delta$ (TGY motif), [21]), and mammalian ERK5 (big MAPK-1, BMK-1 (TEY motif) [22, 23]). ERK5 is unusual because it possesses a long carboxy-terminal extension consisting of a transactivation domain and a nuclear localization signal facilitating translocation into the nucleus upon MAPK activation [8]. Multiple isoforms of MAPKs often exist within individual cells, which can either be activated by different MKKs or can themselves phosphorylate alternate downstream substrates [24]. Additional phylogenetically distinct MAPK subfamilies are defined by categorizing distantly related MAPKs including those from plants (TEY motif), yeasts (T[EN]Y motif), and protozoans (TXY motif, where X is often D or E, but many exceptions exist) [5, 18].

Several atypical MAPK subfamilies also exist, largely representing MAPKs that can only be monophosphorylated within their activation loops. Mammalian ERK3 [25] and ERK4 [8], possessing an SEG motif in the phosphorylation lip and an RXPR motif in the substrate binding pocket, are representative members of one major subfamily of atypical MAPKs, while <u>Nemo-like kinases</u> (NLKs, with a T[HQ]E motif) comprise a second major atypical MAPK subfamily [26]. Greater sequence diversity exists in the phosphorylation lip of atypical protozoan MAPKs (most commonly TGH or TSH motifs) compared to metazoan MAPKs, but members of this subfamily otherwise closely resemble typical MAPKs.

Monophosphorylated human ERK2 has 10- to 100-fold less kinase activity than dually phosphorylated ERK2 [27], illustrating that dual phosphorylation (as is the case for typical MAPKs) achieves greater signal amplification and range of responses than can be achieved by monophosphorylation (as is the case for atypical MAPKs). In addition, different upstream activators can preferentially phosphorylate the threonine or tyrosine within the activation loop of typical MAPKs, allowing signals from two different origins to elicit a response [28]. Typical MAPKs are also subject to a tertiary level of control through the expression of phosphatases specific for either phosphothreonine or phosphotyrosine in the activation loop [29].

Human ERK8 (homologous to rat ERK7) represents a prototypical member of a large atypical MAPK subfamily [30]. Although these large atypical MAPKs contain a TEY

motif capable of dual phosphorylation, activation of mammalian ERK8 (or ERK7) is not under the control of any known MKK family member. Instead, they are activated by autophosphorylation of their activation loops in response to conformational changes in their carboxy-terminal extensions [31]—a highly unusual feature for mammalian MAPKs. Their carboxy-terminal extensions possess a nuclear localization signal that is only exposed in the activated state, thereby facilitating MAPK translocation to the nucleus, which in turn regulates cell proliferation [32].

We performed ClustalW alignment [33] comparing the amino acid sequences of representative metazoan (Homo sapiens [21, 34], Drosophila melanogaster [35], Caenorhabditis elegans [36]) and yeast (Saccharomyces cerevisiae [6]) p38 MAPKs to unique protozoan MAPKs described in this review (Figure 1). Human p38 α was selected as a prototypical MAPK for comparison for three principal reasons. First, a plethora of p38 MAPK inhibitor drugs currently exists [37, 38]. Second, the binding specificity of the pyridinylimidazole p38 MAPK inhibitor SB203580 to the ATP binding pocket of human p38 α is well understood [39, 40]. Third, we have shown that p38 MAPK inhibitors effectively inhibit the in vitro replication of protozoan parasites such as Plasmodium falciparum (Brumlik et al., submitted), L. donovani (Brumlik et al., unpublished observations), and T. gondii [41]. We have further demonstrated that the pyridinylimidazole p38 MAPK inhibitor RWJ67657 protects mice from lethal challenge with T. gondii [42]. Figure 1 demonstrates that while the overall structure of MAPKs is highly conserved even between distantly related eukaryotes, unique features exist that could lead to the design of MAPK inhibitors specific for protozoan parasites.

2. Phylum Apicomplexa

Apicomplexa is a large, diverse phylum comprising over 5000 species, of which seven are known human pathogens (in the genera *Babesia*, *Cryptosporidium*, *Cyclospora*, *Isospora*, *Plasmodium*, *Sarcocystis*, and *Toxoplasma*). There are no reports of functional studies of MAPKs from *Babesia*, *Cryptosporidium*, *Cyclospora*, *Isospora*, or *Sarcocystis* to our knowledge. This section will thus focus on *Plasmodium* and *Toxoplasma*.

2.1. Genus Plasmodium. The genus Plasmodium contains four significant human pathogens, all agents of malaria: *P. falciparum, P. vivax, P. ovale,* and *P. malariae. P. falciparum,* which causes the most severe form of malaria, possesses only two MAPKs. Its Pfmap-1 represents a typical MAPK that is predominantly expressed in gametocytes [43] while Pfmap-2 represents an atypical MAPK (Table 1, Figure 1), which instead possesses a TSH phosphorylation lip [44]. PfPK7, which bears extremely limited homology to mammalian MKK3 and MKK6 that activate host p38 MAPK, does not appear to be a true MKK homologue. Furthermore, PfPK7 is unable to phosphorylate either recombinant Pfmap-1 or Pfmap-2 *in vitro* [45], suggesting that it does not represent a long-sought-after member of an MAPK cascade

		10	20	30	40	50	60
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HS_p38α	Y Q N L S P V C	G S C A Y G S V	/ C A A F D T K T - G L I	R V A V K K L	- S R P F Q S I I HA -	- KRTYR <mark>E</mark> L	RLLKHMK
CE_p38	YINLTPI	G T G A Y G T V	C A A E C T R S - G T I	RVAIKKF	- N R P F Q S I I HA -	- R R T Y R <mark>E L</mark>	RLLRCMC
DM_p38α	Y Q D L Q P V C	G S G A Y G Q V	S K A V V R G T - NMI	HVAIKKL	- A R P F Q S A V H A -	- K R T Y R <mark>E L</mark>	R L L K HMD
SC_Hog1	Y N D L N P V C	GMGAFGLV	CSATDTLT-SQI	P V A I K K I	- MKPFSTAVLA-	- K R T Y R <mark>E L</mark>	KLLKHLR
EH_EhMAPK	Y D I V Q K I C	G K C A Y G V V	WKAVDKTT-HE	Γ V A L K K I	- F D A F Q N A T D A -	- QRTFREI	MYLQRMD
GI_ERK1	Y K V T K A L C	G A G A Y G V V	A E A V D T R T - N T 7	Γναικκι	- SNLFVHLVDS-	- KRTLR <mark>EI</mark>	TILRMLD
GI_ERK2	YEIIHRVC	G K G A Y G V V	WKAVNRKT-NE	Г <mark>V A L K</mark> K I	- F Q A F Q N D T D A -	- QRTFR <mark>E</mark> I	MFLQELD
LMa_MPK1	Y R I L R H I C	G S G A Y G I V	/ WC A L D R R T - G K (CVALKKV	- Y D A F G N V Q D A -	- Q R T Y R <mark>E V</mark>	MLLQRLR
LMa_MPK2	YEIQAQL	GQCAYCI V	WR A L E R K H - N R V	V V A L K K I	- Y D A F Q N S T D A -	- QRTFR <mark>E</mark> I	MFLHRLH
LMa_MPK3	Y T L L K I L C	GM G A Y G - 1	ACSCLDGDTGEI	K <mark>V S I K</mark> K C	- R D V F R D V E D G -	- KRVLR <mark>E</mark> I	DMMRFFH
LMa_MPK4	Y D L V K V V C	G F G A C G T V	C S A V A N G S - G E I	R V A I K R L	- S R V F G D L R E G -	- KRILR <mark>E</mark> M	EIMTSLK
LMa_MPK5	Y T V T S V I C	GH G A Y GV V	CAALDDRT-FQI	E V A I K R V	- S R V F E D L I D G -	- R R I W R <mark>E I</mark>	LLLRILK
LMa_MPK6	YETLGILO	G E G T Y G V V	V K A R S R V T - G K I	LVAIKRF	KQ T E Q D E H V - 1	R K T S S R <mark>E V</mark>	RMLQLLQ
LMa_MPK7	F E V L NG I C	G Y G A Y G V V	C A A V D L R - • - P 1	F V A I K K V	T - K V F D D L V D G -	- R R I L R <mark>E I</mark>	KLLRYLQ
LMa_MPK8	Y D V L E V I C	G E G T Y G V V	F K C R D K R T - N R	I VAVKQF	KN F Q T N A Y V - 1	RVAML R <mark>EL</mark>	RVEQLLK
LMa_MPK9	YTVMGQL	GDGSFGTV	SKAQNTST-GE	I VAVKKM	- KQRFHSWEEC-	L Q L R <mark>E I</mark>	QSLRKVQ
LMa_MPK10	Y T V Q R F I S	S S G S Y GA V	CAGVDSEGII	P V A I K R V	F N T V S D - •	- KRVLR <mark>E</mark> I	RLLNHFH
LMa_MPK11	YLLERIIC	G A G S Y G V V	I R A R D T K SDN R I	L V A <mark>M K</mark> R V	NKEIFEEVILA-	- KRILR <mark>E</mark> I	KLLAHFN
LMa_MPK12	Y N V Q H F V C	G R G A Y G F V	CSAVDAVT - NEI	P V A I K K V	- MHL F DDAVDA-	- KRVLR <mark>E</mark> V	KLLAYLK
LMa_MPK13	Y Q I L G K K C	G E G T F S E V	LRAQDIKT-QQ	Y VAIK CM	- K K A F K S K E Q V -	- N R - L R <mark>E I</mark>	QAVRRLQ
LMa_MPK14	Y E I L A Q I C	GDGTFGSV	A K A V S K K T - G Q I	L V A I K KM	- KQKFYTWEEC-	- V K - L P <mark>E V</mark>	DVVRRIH
LMa_MPK15	Y I L V K Q I C	G K <mark>G</mark> G F <mark>G</mark> A V	EEYTDAIT-EDI	V A I K T I	- P S R Y V N - Q E S -	- R R L V R <mark>E I</mark>	DIMCFLH
PF_Pfmap1	Y D I L K K V C	G K G A Y G V V	F K G R C K K N - K N	I VAVKKI	- F G A F Q N C T D A -	- Q R T F R <mark>E I</mark>	IFLYELN
PF_Pfmap2	YEIKHLIC	G R G S Y G Y V	Y L A Y D K N A - N K I	N V A I K K V	- N R M F E D L I D C -	- KRILR <mark>E</mark> I	TILNRLK
TG_TgMAPK1	F V K K V C	G S G A Y G C V	/ A • - K l	K V A V K K I	- GDL F RDL I DA -	- KRIYR <mark>E</mark> I	KILKELK
TG_TgMAPK2	YDILQKL	GKGAYGIV	WK S T D R R T - N E T	Γ ν αικκι	- F D A F Q N A T D A -	- QRT F R <mark>E I</mark>	MFLQELA
TG_TgMAPK3	YEIRHLIC	GT G S Y GHV	C E A Y D K L E - K R V	V V A I K K I	- L R V F E D L I D C -	- KRILR <mark>EI</mark>	AILNRLN
	* + *	<pre>. * + + * . *</pre>	< + . + +	*+**++	+ . + +	++.++**	. + + + .
		I		I			III

70 80 90 110 100 120 + + + + . + . + + + . + + - - H - ENVIGLLDV- - - FTPARS- - LEEFNDVYLVTHLMGADLNNIVKCQK- - LTDDHVQF HS_p38a - - H - ENIIDLLDV- - - FTPNEN- - VNDIEDVYFVSMLMGADLSNILKIQR- - LNDDHIQF CE_p38 - - H - E NV I GL L D I F H - P H P A N G - S L E N F Q Q V Y L V T H L M D A D L N N I I R M Q H - - L S D D H V Q F DM_p38a - - H - ENLICLQDIF - - LSPLED - - - - - - IYFVTELQGTDLHRLLQTRP - LEKQFVQY SC_Hog1 - - H - ENIVQLVNV- - - MKAENN- - - - - KDIYLAFEYMETDLHAVIRANI- - LEDIQIRY EH_EhMAPK - - H - E NI V K L L D V - - - L V P E D P - - - S N F D D L Y V V F D F MQT DMH K I I S S K Q - D L S P D H M Q Y GI_ERK1 - - H - DNI I RL FNV- - - L KA END- - - - - KD I YL V F E F LDS DL HQV I K SN I - - L ED I HKRY GI_ERK2 - - H - - NP F IVGILD V - I RAAND - - - - - IDL YL V F EL IEADL TAI I RKNL - LQRDHKRF LMa_MPK1 - - H - P NI I KL LHV - H - - RAFND - - - - - RD I YL V F EYMET DLHVV I RAN I - - L E E I HKQF LMa_MPK2 - - H - E NL L NV VN I - - - L P P L K R - E Y H S F E D V Y V V T P L MD V DM N V V L R S R Q - V L E E S HMQY LMa_MPK3 - - H - N NL I RLHHF - - - MRPQSK - - - ETFEDI YL VMDL YDT DLNR I I RSRQ - KLTDEHLQY LMa_MPK4 ECGCRNVLRLIRV- -- LPPRDP- - IMEFRDLYLVTDLYDIDLFSIIRQNK- CESIDLLRR LMa_MPK5 - - H - P NV I R L E D V - - - F R R E G K - - - - - - L Y L V F E F I D Q T I L Q L L E S T T R G L H R R E L R R LMa_MPK6 LMa MPK7 G - H - P NI V RLMEVG RP P A P T G A S S A A F D D I Y L V T D L MD T D L G A L L R S S Q - E I AMD Q L R F LMa_MPK8 S - - EP NVTQLLET- - - FKQKNR- - - - - - VYL VMEY I PR SLLDVLEEVQHGLPED SLVV - - H - PNLVKLKEV- - - VREKTE- - - - - - L FMI F EY CEKNADEM- - - - • - - - E - - I RS LMa_MPK9 - - H - P NI L G L R D I F - - V H F E E P - - - A M H K L Y L V T E L M R T D L A Q V I H D Q R I V I S P Q H I Q Y LMa_MPK10 D - - - DNI I GLRNI - - - LTPKDP - - - ENFDHFYI VMDI METDLKQVLRSGQ - ELTEAHIQF LMa_MPK11 - - H - P NI L S L K D L - - - F K S P D P - - V D T Y S E L Y V V T D L ME S DMDA I L R S P R I R L A A G H G Q Y LMa_MPK12 LMa_MPK13 P - H - P NI V D L V E V - - - L F D R S T - - - - G R L A L V L E L MDM S L Y E L I K G R K Q Y L G E E K V R S G - H - P NVVKLREV- - - I RENNE- - - - - - L F F V F E Y MDG DLLGVIKKA - • - I P Y P LVKN LMa_MPK14 E A H - P HV I GY F S I - - - - • - - - - K T D E F N - V H I VMP L MKG D L F Y F I R L L - • - - - - - - - - -LMa_MPK15 PF_Pfmap1 G - H - DNI I KLMDV - - - I KAKND - - - - - NDI YL I FDF MET DLHEVI KADL - - LEEI HKKY S - - - DYI I RLHDL - - - I I PED - - - LLKFDELYIVLEI ADSDLKKLFKTPI - FLTEQHVKT PF_Pfmap2 - - H - ENI INLVEI - - - LDPLTP - - - DFEDI YL VSDLMDTDLHRVI YSRQ - PLTPEHHQY TG_TgMAPK1 G - H - E NI V R L KNV - - - L KADND - - - - - KD I Y L V F DY MET DL HAV I RADI - - L E E I H K QY TG_TgMAPK2 - - H - DHVVKVLDI - - - VIPKD - - - VEKFDELYVVLEIADSDFKKLFRTPV - YLTELHIKT TG_TgMAPK3 . . + + . . . + . + +

FIGURE 1: Continued.

V

VIa

	130	140		150	160	170	180
	+ . + + + + . + + .	++ *+ ++ ***	* + * + * +	++	+ . ++ . + + 🛛	* . * + + + + *	
HS-p38α	L I Y Q I L RGL K	Y I <mark>h</mark> s adi i hrdi	. K <mark>p s n</mark> l	AV	VEDCELK-	I <mark>L D</mark> F GL A B	HTD
CE_p38	L V Y Q I L RG L K	Y I <mark>H</mark> S ADI I <mark>HRDI</mark>	. K P S N I	AV1	VEDCELK-	I L <mark>D</mark> F G L A <mark>B</mark>	QTD
DM_p38α	L V Y Q I L RG L K	Y I <mark>H</mark> S AGV I <mark>HRDI</mark>	K P S N I	AV 1	NEDCELR-	I L <mark>D</mark> F G L A R	РТЕ
SC_Hog1	<u>FL</u> YQ <u>IL RGLK</u>	Y V <mark>H</mark> S A G V I HR D I	K P S N I	LI1	NENCDLK-	I <mark>C D</mark> F GL A R	IQD
EH_EhMAPK	I I Y Q L L KA L K	Y L <mark>H</mark> S AG I V <mark>HR D</mark> I	. K P S NL	LL1	SDCLLK-	ADFGLAR	S L D - - - - - - - -
GI_ERK1	F V Y Q L L RG L K	Y L <mark>H</mark> S ANC V <mark>HR D</mark> I	. K P S NL	LL1	SDCALE-	I C <mark>d</mark> L G L A R	L V DDHA A KT K
GI_ERK2	I I Y Q CV KAL K	Y L <mark>H</mark> S A E I L <mark>HR D</mark> I	. K P S NL	LL1	SECHMK-N	I A D F G L A R	S I A A L
LMa_MPK1	L T Y Q L L R T V A	QL <mark>H</mark> AQNI I <mark>HRDI</mark>	. K P A NV	F V	S S D C S I K - I	L G <mark>D</mark> F G L A R	T F R S
LMa_MPK2	I IYQLL KTMK	Y L <mark>H</mark> S A E I L <mark>HR D</mark> M	1 K P S NL	LV1	SDCTMK-	ADFGLAR	SIL
LMa_MPK3	FVYQILRGLK	Y L <mark>H</mark> S ANV A <mark>HR DI</mark>	. K P A N L	V T 1	VISCELK-	I I D F G L S R	SVD
LMa_MPK4	FMIQAF RGLH	Y L <mark>H</mark> S A KVM <mark>HR DI</mark>	. K P S NL	LV1	NADCALA-	I C <mark>D</mark> F G L A R	DDQ
LMa_MPK5	I S <u>VR</u> VL RCLA	DM <mark>H</mark> SMG I V <mark>HRD I</mark>	K P S N I	LLR-DEKN	NAE-EVI-	/ C <mark>D</mark> F G L A R	AGL-H
LMa_MPK6	Y T Y Q L L RG I E	F C H <mark>NHNV I</mark> HR DV	K P E NV	LII	D E SG L L K - <mark>I</mark>	L C D F G F A R	QT S
LMa_MPK7	I AYQLMKVLV	Y V <mark>H</mark> S S G V I <mark>H R D I</mark>	K P G N I	LL1	NGNCDMK-I	L C <mark>D</mark> F G L S <mark>R</mark>	G
LMa_MPK8	LLFTILLGIR	SC <mark>h</mark> rngii <mark>hrd</mark> v	K P E N I	L V R I	D - DGAA S - <mark>I</mark>	. C D F G F C R	P L P R
LMa_MPK9	IMCQTL LGVQ	A I <mark>H</mark> KAGFM <mark>HRDI</mark>	. K P E NL	L I S G I	D L - V K - V	/ A <mark>D</mark> F G L A K	EIR
LMa_MPK10	FMYHILLGLH	V L <mark>H</mark> E A GV V <mark>HR D</mark> I	HPGNI	LLA D 1	ND I T-	I C D F NL A R	EDT
LMa_MPK11	F I Y Q AL RALH	I I HSAGVI HRDI	ΤΡΑΝΙ	LV1	NTNCDLK-	I C <mark>d</mark> f g l a k	ЕЕМ
LMa_MPK12	FTLQLLCALQ	Y I <mark>H</mark> S AHV L <mark>HR DI</mark>	. K P G N L	LTI	O S ECN L K - I	L G <mark>D</mark> F G L A R	G I G - H
LMa_MPK13	YMYQLL KGLD	HA <mark>H</mark> R I GV F <mark>HR D I</mark>	K P E N L	LII	DAEGHLK-	I A D F G S C K	GVY
LMa_MPK14	YMRQML QALV	Y I <mark>h</mark> k r g y f hr dn	1 K P E NL	LIR-KEA	S GDEVLK- <mark>I</mark>	ADFGLVK	EIR
LMa_MPK15	FAFQICFGLD	Y L H <mark>Q C F I I</mark> HR DM	1 K P D NV	LVRLDITI	N P YMS T AL	I A D <mark>M G L A R</mark>	DAQ-H
PF_Pfmap1	I IYQLL RALK	Y I <mark>H</mark> S G G L L HR D I	K P S N I	LV1	NSECHIK-	ADFGLAR	SISTH
PF_Pfmap2	I LYNLL LGEK	F I <mark>H</mark> E S G I I <mark>HR D I</mark>	. K P A NC	LL1	VQDCSVK-	I C D F G L A R	T I NSDKD IH I
TG_TgMAPK1	FLYQLL LGLS	F L <mark>H</mark> q a d i i hr di	K P S N I	LV1	VLNCDIK-	I C <mark>D</mark> F G L A R	GLN
TG_TgMAPK2	I VYQLL RAIK	YM <mark>H</mark> SGELL <mark>HRD</mark> M	1 K P S NV	LL1	N S ECQVK-	ADFGLAR	S V A - H S E S
TG_TgMAPK3	L L Y N L L VGV K	Y V <mark>H</mark> S A G I L <mark>H R D I</mark>	K P A NC	LV1	VQDCSVK-	/ C D F G L A R	T V DY P E N GN S
	+ . + + + + . + + .	++ <mark>*</mark> + ++ <mark>***</mark> *	* + * + * +	++	+ . ++ . + + •	• . * + ++ + *	
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VIa

VIb

VII

IX

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	190	200	210	220	230	240
		▼ . ▼ * . * * * * * + *	*+* +	+ + <mark>* * *</mark> + . + +	* +. * +++	*+++.
HS_p38a	D EM	T G Y V A T RWY R A P I	EIM-LNWMH	H Y N Q T V <mark>D I W</mark> S V G C	I MA E L L T G R T	LFPGT
CE_p38	S EM	Г <mark>G Y V A</mark> T RWY R <mark>A</mark> P I	EIM-LNWMH	H Y T Q T V <mark>D V W</mark> S V G C	ILAELITGKT	LFPGS
DM_p38α	N EM	T G Y V A T RWY R A P I	EIM-LNWMH	HYDQTV <mark>DIW</mark> SVGC	I MA E L I T R R T	LFPGT
SC_Hog1	P QM	Г <mark>G Y V S T R Y Y R </mark> A P :	EIM-LTWQF	K Y D V E V <mark>D I W</mark> S A G C	I F A <mark>E</mark> M I E G K P	LFPGK
EH_EhMAPK	KETLQ	Г <mark>DYVE</mark> TRWYR <mark>A</mark> P	EIL-LGSQH	R Y S F A I <mark>D L W</mark> S V G C	I LGE I INGKP	LFPGS
GI_ERK1	ELKDAEKHDTQM	ΓΕΥ <mark>V</mark> ΑΤRWYRΑΡΙ	EII-LGWPC	Q Y G K P V <mark>d i f</mark> s v g c	I F A E L I A R K P	LFPGR
GI_ERK2	SE-PSSATNPIL	T D Y V A T RWY R S P I	EIL-LGCTF	R Y T K G V <mark>DMW</mark> A I G C	I L G <mark>E</mark> M L G G S P	MFPGS
LMa_MPK1	GF-DNEQEFLDL	T D Y I A T RWY R S P I	EIL-VKSRA	A Y S T AM <mark>dmw</mark> a V G C	V I G <mark>E</mark> ML L GH P	LFEGR
LMa_MPK2	SLEGEQASRPVL	T D Y I A T RWY R P P I	EIL-LGSTF	R Y T K G V <mark>DMW</mark> S V G C	I L A E L M L G K P	IFPGR
LMa_MPK3	- V P Y S E L	T D Y V I T RWY R P P I	EL <mark>L</mark> - LENTN	J Y S T A V <mark>D I W</mark> S V G C	I F A <mark>E</mark> MY N R K P	VFPGR
LMa_MPK4	- V M S S S D L	T Q Y V V T RWY R P P :	E V L GMG S N C	Q Y T S A V <mark>D V W</mark> S L G L	I F A E LMV G R A	LLPGT
LMa_MPK5	RLSEPLDL	T D Y V V T RWY R P P :	E L L - LMC P -	Y S Y P I <mark>d I w</mark> a v g c	VMA E Y AMQ R P	LFAGR
LMa_MPK6	A R G K Y	T D Y V A T RWY R A P I	ELL - VGDVA	A Y G K P V <mark>D V W</mark> A L G C	M F A <mark>E</mark> L S D G Q P	LFPGE
LMa_MPK7	• L Y S L	Г D Y V V T R Y Y R A P :	E L L - I - MG F	R Y NHA I <mark>DMW</mark> S A G C	I L A EMV L R R P	LFTGA
LMa_MPK8	• A I M	Г <mark>NYVA</mark> TRWYRSP	EM <mark>l</mark> - lgms s	S Y T Y A V <mark>DMW</mark> A V G A	I MA E A I D G E P	LLPGK
LMa_MPK9	S R P P F	ΓΕΥ <mark>V</mark> STRWYRAP	EL <mark>V</mark> - LHSTH	H Y N S P V <mark>D I W</mark> A C A V	I F A E L Y L C R P	LFPGT
LMa_MPK10	ADANK	T H Y V T H R W Y R A P I	E L <mark>V</mark> - MQ F K (G F T K L V <mark>DMW</mark> S A G C	VMA <mark>E</mark> MFNRKA	LFRGS
LMa_MPK11	DQGEYM	T D Y V T MRWY R A P I	E L <mark>v</mark> - medki) Y S V Q I <mark>D VW</mark> G I G C	I L G E L L G S R P	LFQGK
LMa_MPK12	DDTM	T Q Y V F T RWY R P P I	ELL - LVCKH	I C N Y S A <mark>d Mw</mark> a V G C	L A A <mark>E</mark> M F T G K P	LFPGK
LMa_MPK13	S K L P L	ΓΕΥΙSTRWYRAP	ECL - LTDGY	Y Y N Y KM <mark>D L W</mark> S A G C	V F F <mark>E</mark> I I A L F P	LFPGS
LMa_MPK14	A R P P F	T D Y V S T RWY R A P I	ELL - LQDRH	F Y G A A V <mark>D V W</mark> A A G C	IMVELITMRP	LFPGT
LMa_MPK15	SD	Г I Y I C T R Y Y R P P I	E V I - T S V S C	G G S P R I <mark>D I W</mark> S L G C	I F Y <mark>E</mark> MC T G Q T	LFTMR
PF_Pfmap1	- V NENKVPIL	Г D Y V A T RWY R A P I	EIL-LGSTH	H Y T E D V <mark>DMW</mark> S L G C	I MG E L L C G K P	LFTGN
PF_Pfmap2	• - NKNLKKQL	Г S HVV T RWY R A P I	ELI-LLQEN	JY TN S I <mark>d I w</mark> S T G C	I F A E L L - • - P	LFPGS
TG_TgMAPK1	• DMEL	T D Y V V T RWY R P P 1	EIL - ISPFO	C Y S K P V <mark>D L W</mark> S V G C	I F A E L L G R R A	LFAGK
TG_TgMAPK2	NN - SEAGGNPVL	T D Y V A T RWY R A P I	EIL-LGSTS	S Y T K G V <mark>DMW</mark> S L G C	I L G E L L S G R P	IFPGT
TG_TgMAPK3	• L K R Q L	T G H V V T R W Y R A P I	ELI - LLQEN	JYTEAI <mark>dvw</mark> sigc	I F A E L L - • - P	LFPGS
0		▲ . ▲ * . * * * * * + * *	*** +	$+ \dots + * * * + \dots + + * * * + \dots + + * * * *$	* + . * + + +	*+++.

VIII

FIGURE 1: Continued.

		250	260	270	280	290	300
	+ +	*+++	×	* + + +	+		
HS-p38a	DHID-	QLKLILRLV	G T	PGA ELL	K K I S S E S A R N -	Y I Q S L -	- TQMPK
CE_p38	DHID-	QLTRIMSVT	G T	PDE EFL	K K I S S E E A R N -	Y I R N L - 1	РК - МТ К
DM_p38α	DHI-H	QLNL IMEML	G T	P P A E F L	K K I S S E S A R S -	Y I Q S L -	- PPMKG
SC_Hog1	DHV - H	QFSIITDLL	G S	P P K D V I	NTICSENTLK-	FVTSL - 1	PH-RDP
EH_EhMAPK	S T L N -	Q L D K I I E A T C	G Q	P S A E D L	E V I D S P L S MN -	L L S S L - 1	P Q - R E T
GI_ERK1	DYI - H	Q L H L I L E V L (G T	PEKELL	DRIASDSAKS-	YVLAL -	- K P S A P
GI_ERK2	S TMN -	QLDKIM(G G - HWE R	P T P E D I	EATESPFASM-	MLDSL -	- Q P K T G
LMa_MPK1	NTLD-	QLRLIVEAI	6 V	P S D A D V	R S L H S P E L E T -	L I N S L -	P T P
LMa_MPK2	S T T N -	QLELICSVT	G M	PSAADV	AATNSQFAHA -	MLRDI -	- HCAHR
LMa_MPK3	NTMD -	Q L RM I AQH I (G K	P P A	SIVEHREALE -	K L N E L -	PDGS
LMa_MPK4	DYI - G	QLVMIVNLL	G S	P SI DDM	EFLSSE-AKA-	F I L S Q - 1	PH-RPA
LMa_MPK5	DYI - H	Q L Q F V L 8	S S I	P I T G V D F I	ERSSSSGLA-	NMNEIA	KKYKGT
LMa_MPK6	S D L D -	QLCLIMQTC	3	P V P	QRLVFIFMHN-	PLYNGISF	PHTDIL
LMa_MPK7	N Y L - S	QLALILETP	G L R G - V P Q T	PEEAAA	L F E G G E E G K H -	• DP	L T L S DQ
LMa_MPK8	T E L - E	Q L S L I Q T R I (G D F	PAA-• L	N P L A A P P Q Q L -	R T K S MQ	Q K S R R A
LMa_MPK9	S E S D -	Q	G S	P A P N E W	D E - G Y Q L A R R -	MNMR F	PT-VAP
LMa_MPK10	T F Y N -	QLNKIVEVV	G T	PKI EDV	VMF	Y L R N S - 2	LSNVPA
LMa_MPK11	DRVN-	QLDKIVDVI	G T	P S E E D I 1	N S V G S S A A Q K -	Y L K K K -	- SHRPQ
LMa_MPK12	DY I N -	QINLIVELL	•	PSKG-KKL	EEYAPELRRR	E D E T T F Y D S F D'	ΓΕLΕΕΑ
LMa_MPK13	NELD-	QVHR I HNV L	G T P	P T E I L E R L	K K F G T HMD Y D -	F	PK-KQG
LMa_MPK14	NEVD-	Q L F K I M S V L C	G S	P T E E V	WAGGLRLAKK -	I R Y T F	PK-VAG
LMa_MPK15	- •	Q L E V V L N T I C	G T	PAAEDI	E R YMP S GNAK -	L Y L Q R S -	A A R P
PF_Pfmap1	S TMN -	QLEKIIQVI	G K	PNK KDI	E D I R S P F A E K -	I I S S F -	- VDLKK
PF_Pfmap2	- • - D -	Q L N I I F N V I (G T	P P E E D L	КСІТКQЕVІКУ	IKLFPTRDGI	D L S K K Y
TG_TgMAPK1	DHFD-	QLRRIVRVL	•	P S K G T S N T	K R K R S E A A R R -	F I E S L - 1	PN-SDP
TG_TgMAPK2	S TMN -	QLERIMTLT	G R	P S P E D V	DAVKSPFAAT -	MMESL -	- PLGKV
TG_TgMAPK3	- • - D -	QLNVIFNILO	G T	P S E E D I	EALEKEDAKRY	IRIFPKREGTI	DLAERF
-	+ +	* + + + *	K	* + + +	+		

Х

	310	320	330	340	350	360
		+.	++++.+++.	+++.+*.*	+ . + . + . + + + +	+ + +
HS_p38α	MNFA - NVFIGA	N P L A -	V D L L E KM L V	LDSDKRIT	A A Q A L A H A Y F A Q Y	HDPDDE
CE_p38	RDFK-RLFAQA	T P Q A -	IDLLEKMLH	LDPDRRPT	A K E AMEHEYLAAY	HDETDE
DM_p38α	RSFK-NVFKNA	N P L A -	I D L L E KML E	LDAEKRIT	A E E A L S H P Y L E K Y	A Ê P S V E
SC_Hog1	I P F S - E R F K T V	E P D A -	V D L L E KML V	FDPKKRIT	A A D A L A H P Y S A P Y	HŨPTDE
EH_EhMAPK	KGLA - E I V P K A	SDDA -	LELMEELLT	FNPEKRAT	A E K A L E S T F V A D F	HDPNDE
GI_ERK1	QDLS-QKFPML	DEAG	I D L L T RML T	LDPLK <mark>R</mark> IT	VNECLSHPYFEGI	HDESDE
GI_ERK2	K A L S - E I Y P N A	P A D A -	LDLLKKLLQ	FNPNKRLT	A E Q A L E H P Y L S K F	HDPATE
LMa_MPK1	L I F S - P L V G N K	- SLKDSEAT	- DLMMKLIV	FNPKR <mark>RL</mark> S	A V E A L Q H P Y V A P F	LQPGEL
LMa_MPK2	R T F A - E L L P S A	SADA -	LDLIERFMR	FNPNRRIS	AAEALEHPYVAAF	HRPDEE
LMa_MPK3	L N I P - K L V P G L	A G N T E G -	I D F L S KMWT	LDPSKRPT	AADMLAHPYLAHL	HDEEDE
LMa_MPK4	LSFR-DLFSMA	T E E A -	TDLLSKLLV	FHPARRLT	A K Q V M E H P Y F S K Y	RĎAĂĚE
LMa_MPK5	R P L P - Q L L S K L	P R D G -	LELVTEMLA	FEPNKRIT	AQEALKHPFFSSV	GĞPDĈK
LMa_MPK6	Y T L K - E R Y H R E	SNDW-	LEFLSSCLH	T D P A Q <mark>R</mark> L <mark>T</mark>	CTELMELPYFTRD	GFRDRY
LMa_MPK7	VHSQ-VLFHSTLFGF	KVDVPISLG	I - LIAKLLS	FDPRKRPT	A L E A L R D P F F W P L	YDSRDE
LMa_MPK8	S D V - • - R Y G G R	I A K A G	LNLLHGLLR	IDAAERIT	V E E A L G H P Y F D S V	RGRFDA
LMa_MPK9	T P L R - H I L T T A	P P A A -	V D L M A Q M L R	FNPAERPT	ATQCLQHPYFTGS	GGSSÄL
LMa_MPK10	R AWT - A V V P T A	D P V A -	L D L I A KM L E	FNPQR <mark>R</mark> IS	T E Q A L R H P Y F E S L	FDPLDL
LMa_MPK11	A DWR - Q R Y P K A	S P E A -	LDLLRHMLV	FNPKRRIT	V L Q A M R H P F L E Q L	HDDADD
LMa_MPK12	IAVEGAIIARPRPHP	PEEYYAEF -	VDFIFGLLC	Y N P A K <mark>R</mark> R T	AKESIAHAWLSDV	RGPQET
LMa_MPK13	T G L G - K L L P H V	S A E A -	LDLMKKLLT	Y D E E Q <mark>R</mark> C T	A K E A L R H A Y F S K L	READKK
LMa_MPK14	SGLA - QALPSH	I P L P A -	LDLLRQMLV	Y D P K V <mark>R L</mark> T	A E Q C L Q H P F F N V G	IĎEĈNA
LMa_MPK15	SQLR-QLIEQNWILH	I T S A D E K E KW	IDLITRCVA	FFPEQRPT	AQQLCQHQLFRNY	NVFYGS
PF_Pfmap1	K N L K - D I C Y K A	S N E S -	LDLLEKLLQ	FNPSK <mark>R</mark> IS	A E N A L K H K Y V E E F	HSIIDE
PF_Pfmap2		S S I S K E G	IDLLESMLR	FNAQKRIT	IDKALSHPYLKDV	RKENÏE
TG_TgMAPK1	Y K L E - D L F P D A	S K A A -	LDLLSNLLT	FDPAKRIT	V Q E A L R H E Y F E G L	HSVEDE
TG_TgMAPK2	K N F K - D A F P N A	S P E A -	LDLLKQLLQ	FNPNKRIS	A E K G L E H P Y V R Q F	HSPÉĎE
TG_TgMAPK3		PASSADA	IHLLKRMLV	FNPNKRIT	I N E C L A H P F F K E V	RIAÊVE
		+.	++++ . +++ .	+ + + . + * . *	+ . + . + . + + + + +	+ + +

XI

CD

		370	380	390	400	410	420	C-terminal
								Extension
US p38a	DVADDV	DOSEESI		TVDEVISEVE	PPIDOF			none
CE p38	DIAEEM			LINEEISDEOR	NVAEAD			none
$DM p38\alpha$	OTSPRV			LI IVEEI SDFQK	PPSVAO			none
SC Hog1				MMVSEIIDEUV				none
EH EhMAPK	PSAPCKI		JHKVSINNVE	CSIVVFIMPKV	PON			none
GLERK1	PVYTGOR		VELTKPLIE	MCEINEARKEH	I QIQ I PDFI KDFVA	KRAKALGIPES	VWM	none
GLERK2	PSAPGPI	IKISIDDI	DKRSVSFYR	DLLYSELLRKK	KEIRSKSLK	FDF	* *****	none
LMa_MPK1	EKIOGLE	I R I U I D D I	DEKIYTKEE	YKANLYDEIGN	IR Y R H H I T D V	Y		none
LMa_MPK2	AVAPEPI	TVSLPD	SORLPLAKYR	DALYEOLAALR	RSSTSADOR	ORAEROTAGST	ASRKTS	60 aa
LMa_MPK3	PACSCPI	FLWAHEST	PMGV S E L R R	A FWADI VDYNP	SLEOATPPV	TTAGGSSSKNG	SGHHH	none
LMa_MPK4	ADAPDPI	FVWNHSH	ETKEOLREE	LWRVVEAHSOL	NE	111100000011110	0 011111	none
LMa_MPK5	SYPAPPE	ELDLGFDN	MHAEVSECOL	RRAIWDELOYY	RKO			none
LMa_MPK6	EAELQAA	AMGLPQLI	RSTPTTSAPS	TORRAPDOAAA	LGDDLKADT	VVSPHKCRSSE	IISPKL	751 aa
LMa_MPK7	ILRCPAS	SDPSVÄRI	EQIDDIAAYC) KAHPCVVVDES	P V F TWE F DH	RITSAQALRGL	FEEECO	48 aa
LMa_MPK8	TANGARE	RVCNSNG	ACDEAADMRF	, TTAETAE SMLM	IPLTTAASRO	APLPPLTATGA	VDCTSP	1,117 aa
LMa_MPK9	YAGIATO	GQPHNPFO	QMAASSAVAA	QSMSNVGLTSN	ISSPPPTTSN	A S L F K Y A N L F N	QGNRSP	49 aa
LMa_MPK10	TEGLSEF	RFHFDESV	TDVYDMHK I	FTAEVERFNDL	RERREEVAR	ERAVAAQQQGE	QVLGTD	19 aa
LMa_MPK11	NLSYTLH	FRFDENEC	QKTIMDVKRA	IYKESVKFHNE	HPSSMRATT	MY S A F N T P S V A	APSVAT	29 aa
LMa_MPK12	IGGCEAH	E R I Y RWDA	ADGTAFTIPC	QLRQLFIDEIGK	FASTRSS			none
LMa_MPK13	SHRLKHS	SASISRPT	T V T D D A H A S	IGLGSSPRKTT	'NGMP S L N S T	LTASRKLPVID	G K S P T K	36 aa
LMa_MPK14	PSAAALI	DQLALMAH	KRML P G S K T A	P P A L K S P T T D Q	VAALKAARA	STDASLSDTSS	R K F Y L L	288 aa
LMa_MPK15	NVKQYAI	PTPFTLSY	SGSSDSTRA	ENKAAILALVQ	RALRKTMPQ	RSEESSDEESS	S L N S S S	547 aa
PF_Pfmap1	PTCRHII	I T I P I NDI	NTKYRVNFYR	NVVYFVIMRRN	IK F H S N V L N Q	GESKKEEKKDR	YYRRDK	455 aa
PF_Pfmap2	NFSTEKI	IILPFDDV	VMVLSETQLF	XY I F L K E I Q S F H	IADLIIPAKL	N I HQK S F Y NM		none
TG_TgMAPK1	PTVTTPV	VNWS FDN I	F V P T K R					depends [†] ; none
	PTVTTPV	VNWS FDN I	F V P T K R <i>L L QN</i>	IKVYQE I I SYHP	EIVLRDFHL	LPPRGIHVAPS	S L P A C I	; 723 aa
TG_TgMAPK2	PVCGKII	IAIPIDD	NTKYSVEDYR	RDKVY S EV I KKK	HDQRRHRTA	GSSGRHHSSHH	S S G T R S	308 aa
TG_TgMAPK3	TNATEKV	V R L P F N DV	VMNMD E P Q L F	X Y A F V K E I Q R Y H	IPEIQLPRRS	PNRAS S		none

[†]C-terminal extension is only present on splice variant of *tgMAPK1* containing exon 8.

FIGURE 1: ClustalW alignment of representative MAPKs of diverse evolutionary origin, with each of the 11 subdomains indicated (Roman numerals). Conserved acidic residues within the ED site (subdomain VII) and common docking (CD) domain, which immediately follows subdomain XI, have been underlined. The first four sequences represent p38 MAPKs of metazoan and yeast origin and are boxed as reference sequences to which other protozoan MAPKs can be compared. Invariant MAPK residues (within allowed substitution groups) are highlighted in black and denoted by an asterisk. Highly conserved residues (>80% conservation) are highlighted in grey and denoted by a plus sign. In the absence of grey shading, plus signs indicate residues conserved in the majority of aligned sequences. Allowed substitution groups include acidic/amide (DE, DN, EQ), aliphatic (LIVM), aromatic (FYW), basic (KR), and hydroxyl/polar residues (STG). The positions of insertion sequences removed prior to ClustalW alignment are indicated by filled circles. White triangles denote the position of the TX[XY] phosphorylation lip. Two letter abbreviations precede the name of each MAPK sequence, indicating the genus and species of origin for each MAPK. CE: *Caenorhabditis elegans*; DM: *Drosophila melanogaster*; EH: *Entamoeba histolytica*; GI: *Giardia intestinalis*; HS: *Homo sapiens*; LMa: *Leishmania major*; PF: *Plasmodium falciparum*; SC: *Saccharomyces cerevisiae*; TG: *Toxoplasma gondii*. Accession numbers of all aligned sequences are listed in Tables 1 and 2.

in *Plasmodium*. Moreover, no *P. falciparum* MKK genes have been identified, suggesting that *P. falciparum* MAPK signaling does not utilize typical MAPK cascades [46]. *P. falciparum* Pfmap-2 is instead activated by Pfnek-1, a <u>never-in-mitosis/Aspergillus-</u> (NIMA-) related kinase [47]. Since homology amongst MKKs and MKKKs is much lower than that for members of the MAPK superfamily, it is conceivable that genes encoding these proteins exist but have simply not been annotated as such in the *P. falciparum* genome.

Pfmap-1 is neither required for schizogony nor gametocytogenesis in human erythrocytes cultured *in vitro*, nor for gametogenesis and/or sporogony in the mosquito vector [48]. However, Pfmap-2 protein levels are elevated in *pfmap-1* knockout parasites, suggesting that Pfmap-1 fulfills an important function necessitating compensatory adaptation in parasites lacking this enzyme. Pfmap-2 is essential for the completion of the *P. falciparum* asexual cycle [48]. Functional characterizations of MAPKs from *P. vivax*, *P. ovale*, and *P. malaria*, the other *Plasmodium* species causing malaria, have yet to be reported to our knowledge.

2.2. Genus Toxoplasma. T. gondii, the sole member of the genus Toxoplasma, can cause significant morbidity or mortality in hosts with compromised cellular immunity. Like *P. falciparum*, *T. gondii* appears to be another protozoan parasite that lacks typical MAPK activation cascades. Preliminary examination of the *T. gondii* genome suggests that it encodes four MAPKs. However, the TGME49_021550 locus (situated on chromosome II) lacks coding sequences corresponding to several essential MAPK motifs (including an incomplete MAPK signature sequence), thereby disqualifying it as a functional MAPK gene. Of the remaining three MAPK genes (Table 1, Figure 1), we have cloned and sequenced both the genes encoding *tgMAPK1*, situated on chromosome XI [50], and *tgMAPK2* (chromosome VIII;

7

Organism	МАРК	Accession no.	Phosphorylation lip	Classification	Function	References
Caenorhabditis elegans	p38	AAB00664	TGY	Typical	Stress-response	[36]
Drosophila melanogaster	p38α	AF035547	TGY	Typical	Stress-response	[35]
Entamoeba histolytica	EhMAPK	AY460178	TDY	Typical	?	
Ciardia intestinalis	ERK1	AY149274	TEY	Typical	Encystation	[49]
Gurum miestinuits	ERK2	AY149275	TDY	Typical	Encystation	[49]
Homo sapiens	p38α	Q16539	TGY	Typical	Stress-response	[21, 34]
Dlarmadium falsiparum	Pfmap-1	Q94656	TDY	Typical	?	
r usmoaium juiciparum	Pfmap-2	Q25917	TSH	Atypical	Essential for differentiation	[48]
Saccharomyces cerevisiae	Hog1	AAA34680	TGY	Typical	Stress-response	[6]
Toxoplasma gondii	TgMAPK1 (BARKY)	AY684849	TDY	Typical	Proliferation [†] , differentiation [†] , virulence [†]	
1 0	TgMAPK2	DQ115400	TDY	Typical	?	
	TgMAPK3	XP_0022369585	TGH	Atypical	?	

TABLE 1: Non-Trypanosomatid mitogen-activated protein kinases discussed in this review.

[†]Brumlik et al., submitted.

[18]). We have also sequenced the third MAPK gene, *tgMAPK3* (chromosome Ib).

TgMAPK1 is a critical virulence determinant during acute *T. gondii* infection (Brumlik et al., submitted). By expressing it in Hog1-deficient yeast lacking its own stress-response MAPK, we restored yeast ability to grow under osmotic stress [50], providing evidence for this MAPK's role as a stress-response MAPK. Since TgMAPK1 expression affects tachyzoite/bradyzoite stage differentiation (manuscript in preparation), we renamed it "BARKY" (bradyzoite antigen regulator, kinase \underline{Y}).

BARKY is a typical MAPK based on conventional criteria [50] although it possesses three insertion sequences. Using mass spectroscopy, we confirmed the presence of a 34 amino acid insert situated between the GXGXXGXV motif (subdomain I) and the invariant lysine residue within the VAXK motif of subdomain II, a region responsible for anchoring the nontransferable α - and β -phosphates of ATP during catalysis. *BARKY* is also predicted to encode a 93 amino acid insert situated between the DFGLAR motif that interacts with the Mg⁺⁺ bound to ATP and the phosphorylation lip, which links the proline-directed peptide binding pocket in an extended conformation following phosphorylation of its activation loop (subdomains VII and VIII, resp.). Finally, using mass spectroscopy, we identified a 20 amino acid insert between subdomains IX and X.

Phylogenetic analysis demonstrates that *T. gondii* BARKY most closely resembles *Cryptosporidium hominis* MAPK (with 52% amino acid sequence identity across all 11 of the MAPK subdomains), with a corresponding homologue in *C. parvum.* No other closely related MAPK homologues were identified either within or outside the phylum Apicomplexa at the time of publication [18].

Alternative splicing within exons 3-4 and exons 7-8 of the *BARKY* gene results in multiple BARKY isoforms, producing protein variants that could differentially respond

to upstream signals or have altered substrate specificity. In support, we have detected 50, 58, and ~130 kDa proteins in *T. gondii* tachyzoite cell-free extracts by Western blotting. We have also employed mass spectroscopy to detect peptide fragments that confirm the existence of the full length (130 kDa) BARKY protein in tachyzoites grown *in vitro*. We cannot exclude the possibility that the smaller forms of the protein result from proteolytic degradation, but reverse transcriptase-polymerase chain reaction has demonstrated the presence of *BARKY* transcripts with a stop codonsituated 84 nucleotides into exon 7, as well as an alternative *BARKY* splice variant encoding exon 8 that adds a 766 amino acid extension to the carboxy-terminus (Brumlik et al., unpublished observations). These features are reminiscent of extensions identified in many *Leishmania* MAPKs [51].

There are no reported functional data for T. gondii TgMAPK2 but it is expressed in T. gondii tachyzoites at the expected molecular weight of 73 kDa (Brumlik et al., unpublished observations). Phylogenetic analysis places this MAPK in a group of closely related Apicomplexan MAPKs which includes Cryptosporidium hominis MAPK1, P. falciparum Pfmap-1, and Theileria annulata MAPK (all sharing roughly 70% amino acid sequence identity across all 11 of the MAPK subdomains). TgMAPK2 shares significant amino acid sequence identity with MAPKs from non-Apicomplexan protozoans including L. mexicana LmxMPK2 (62%) and Trypanosoma brucei TbMAPK2 (62%), each possessing a typical TDY phosphorylation lip. The deduced amino acid sequence of TgMAPK2 shares 55% identity with human ERK8 across all 11 MAPK subdomains, demonstrating the remarkable evolutionary conservation of this MAPK subfamily member. In addition, T. gondii TgMAPK2 possesses multiple copies of a VSSSHHG repeat in its carboxyterminal extension, the exact number of repeats being straindependent [18]. While the role of this repeat remains unknown, it is striking that P. falciparum Pfmap-1 possesses an analogous series of imperfect KKYVD[GSE][GSL]N repeats in its carboxy-terminal extension [43]. Short amino acid repeats often facilitate oligomerization or serve as contact points for protein-protein interactions. Interestingly, TgMAPK2 is also predicted to possess a nuclear localization signal within its carboxy-terminal extension.

T. gondii TgMAPK3 is predicted to be an atypical 63 kDa MAPK with a TGH phosphorylation lip. It shares significant amino acid sequence identity with several Apicomplexan MAPKs such as *Cryptosporidium hominis* MAPK2 (67%), *P. falciparum* Pfmap-2 (58%), and *Theileria annulata* MAPK2 (50%), with low amino acid sequence identity to non-Apicomplexan MAPKs [18].

3. Phylum Sarcomastigophora

3.1. Trypanosomatid MAPKs. Trypanosomatids (members of the family Trypanosomatidae) are a diverse group of protozoan parasites of which two genera are human pathogens: *Trypanosoma* and *Leishmania*.

3.1.1. Genus Leishmania. Several different Leishmania species cause human disease of varying clinical presentation and severity, of which *L. major* generally causes the most serious illnesses. Genome sequencing has identified 15 putative complete MAPK genes in *L. major* (Table 2), the alignments of which are shown in Figure 1. Two partial *L. major* MAPK genes have also been identified (LmjF03.0210 and LmjF13.07800) [52] but have been excluded from further consideration because they lack the coding region for the complete MAPK signature sequence. All 15 *L. major* MAPK homologues have also been identified in *L. major* MAPK homologues have also been identified in *L. major* MAPK homologues have also been identified in *L. maxicana* (Table 2), *L. infantum*, and *L. brasiliensis* [52].

Each of the 15 unique *Leishmania* MAPKs (Figure 1) is a typical MAPK by the classical definition (*i.e.*, the activation loop is comprised of a TXY motif). The majority of these MAPKs possess carboxy-terminal extensions (Figure 1), some of them over 1000 amino acids long (as for LmaMPK8). This region may be analogous to the corresponding region of human ERK5 or ERK8, each of which possesses a C-terminal transactivation domain and nuclear localization signal [22, 23]. LmaMPK6, 7, and 8 are predicted to contain nuclear localization signals within their carboxy-terminal extensions, making them even more closely resemble human ERK5 and ERK8, as well as *T. gondii* TgMAPK2.

Deletion analysis of the genes encoding *L. mexicana* LmxMPK1 and LmxMPK2 demonstrates that both are essential for amastigote (bloodstream stage) survival [52, 53]. *L. mexicana* LmxMPK4 is essential to both promastigote (sandfly stage) and amastigote forms [58] and is phosphorylated on T^{190} and Y^{192} of its phosphorylation lip by the MKK LmxMKK5 [64]. Overexpression of *L. major* LmaMPK4, 7, or 10 (homologues of LmxMPK4, 7, and 10, resp.) causes stage-specific induction of phosphotransferase activity. Moreover, LmaMPK7 activation specifically regulates parasite growth [62]. In each case, kinase activity was low or absent in cell-free extracts from promastigotes but significantly increased after exposure to pH 5.5 and 34°C.,

which simulates the stress encountered by the parasite in the acidified phagolysosome upon invasion of macrophages [59]. *L. mexicana* LmxPK4 is an MKK that controls parasite differentiation [65] and thus represents a potential upstream activator of at least one of the MAPKs affecting stage differentiation.

Several *L. mexicana* MAPKs regulate flagellar length, many of which possess carboxy-terminal extensions [66]. Deletion mutants for LmxMPK3 had shortened flagella and overexpression of LmxMPK3 in the deletion background complemented this defect [56, 57]. Deletion mutants for LmxMPK9, LmxMPK13, or LmxMPK14 generated promastigotes with elongated flagella, an effect that could be reversed by overexpressing these MAPKs in null mutants [57, 63]. LmxMPK13 is the homologue of LF4 from the protozoan microalga *Chlamydomonas reinhardtii*, which also regulates flagellar length [67]. *L. mexicana* LmxMKK is the MAPKK responsible for regulating flagellar length [68] and activates LmxMPK3 [56] and perhaps affects other MAPKs regulating flagellar length.

Analysis of the *L. mexicana* genome has identified two additional putative MKK genes in addition to *L. mexicana lmxPK4*, *lmxMKK*, and *lmxMKK5* for which functions have yet to be determined. *L. mexicana* also putatively encodes 23 MKKKs and a single MKKKK [51], the functions of which remain unknown.

3.1.2. Genus Trypanosoma. Subspecies of T. brucei cause African sleeping sickness, whereas T. cruzi causes New World trypanosomiasis (Chagas disease). Genomic sequencing has identified 13 MAPK genes in T. brucei, each of which has at least one, but often two virtually identical copies of MAPK homologues in T. cruzi (with each copy having greater than 99% amino acid sequence identity to the other (Table 2)) [51, 52]. Homologous T. brucei or T. cruzi MAPK domains are ~90% identical to each other and each has a single corresponding homologue in Leishmania spp. (sharing over 80% amino acid sequence identity across the 11 MAPK subdomains). LmxMPK7 and LmxMPK8 are the only two Leishmania MAPKs that lack homologues in either T. brucei or T. cruzi. Thus we exclusively used the L. major MAPK sequences (LmaMPK1-15) for ClustalW alignment, reducing redundant examples of highly homologous MAPKs in the analysis.

Although all *Trypanosoma* MAPKs possess a classical TXY motif, a feature also conserved in all *Leishmania* MAPK homologues (Table 2), the central amino acid in the TXY motif varies between MAPK homologues from different Trypanosomatid species (Table 2) and thus is not as evolutionarily constrained as in mammalian MAPKs.

T. brucei/cruzi MPK10 (accession nos. Q580Z7/ Q4D4Q4) and MPK11 (accession nos. Q389D8/Q4CZQ7) have not yet been officially named (see Table 2). Regardless, these MAPKs (and their *Leishmania* MAPK homologues) are exceptional in possessing a MAPK signature sequence that deviates with respect to the precise position of the threonine in the proline-directed (P+1) peptide binding pocket (see Figure 1, center of subdomain VIII, residues 197-207). This likely alters the precise spatial orientation

	References	[52–55]	[52, 53]	[56, 57]	[52, 58, 59]	[60]	[61]
	Function	Essential for intracellular parasite survival of bloodstream stage (LMx, TB), IFN-y-induced proliferation of bloodstream stage (TB)	Essential for intracellular parasite survival of bloodstream stage (LMx, TB)	Flagellar length (LMx)	Stage-specific induction of phosphotransferase activity(LMx)	Differentiation (TB)	Proliferation; stage-specificinduction of phosphotransferase activity (TB)
s in Trypanosomatids.	Phosphorylation lip LMa/LMx/TB/ TC#1/TC#2	TDY/TDY/ TEY/TGY	TDY/TDY/TDY/ TDY/TDY	TDY/TDY/ TDY/TDY	TQY/TQY/ TDY/TEY	TDY/TDY/ TDY/TDY	TDY/TDY/ TEY/TDY
g homologues	TC ^a accn. #2	Ι	Q4CR01	Q4CKS6	I	Q4DCP6	
corresponding	TC ^a accn. #1	Q4CSB9	Q4CZ09	Q4D0A7	Q4D3Y2	Q4DHF7	Q4DD40
ises and their	T. cruzi (TC) MAPK	I	I	TcMPK3	I		I
protein kina	TB ^a accn.	Q26802	Q38B88	Q580X5	Q38B88	Q586Y9	Q381A7
en-activated	T. brucei (TB) MAPK	KFR1			TbMAPK2	TbMAPK5	TbECK1
[ABLE 2: Mitog	LMx ^a accn.	Z95887	AJ293280	AJ293281	AJ293282	A]293283	AJ293284
	L mexicana (LMx) MAPK	LmxMPK1	LmxMPK2	LmxMPK3	LmxMPK4	LmxMPK5	LmxMPK6
	LMa ^a accn.	Q4Q0B0	Q4Q204	Q4QHG6	Q4QD66	Q4Q701	Q4Q4U7
	L. major (LMa) MAPK	LmaMPK1	LmaMPK2	LmaMPK3	LmaMPK4	LmaMPK5	LmaMPK6

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L. major (LMa) MAPK	LMa ^a accn.	L mexicana (LMx) MAPK	LMx ^a accn.	T. brucei (TB) MAPK	TB ^a accn.	T. cruzi (TC) MAPK	TC ^a accn. #1	TC ^a accn. #2	Phosphorylation lip LMa/LMx/TB/ TC#1/TC#2	Function	References
LmaMPK7	Q4QFZ0	LmxMPK7	AJ293285	I	l		I	I	YUY/YUT	Proliferation; stage-specificinduction of phosphotransferase activity (LMa)	[59, 62]
LmaMPK8	Q4Q8L2	LmxMPK8	AJ293286		I			I	XNT/YNT	~.	
LmaMPK9	Q4QDK3	LmxMPK9	AJ293287		Q387N8	I	Q4DYK0	Q4DD15	TEY/TEY/ TEY/TEY	Flagellar length (LMx)	[57, 63]
LmaMPK10	Q4QHJ8	LmxMPK10	DQ308411	I	Q580Z7		Q4D4Q4	Q4CU32	ТНҮ/ТНҮ/ТНҮ/ ТНҮ/ТНҮ	Stage-specific induction of phosphotransferase activity (LMa, LMx)	[59, 62]
LmaMPK11	Q4Q449	LmxMPK11	DQ026027		Q389D8		Q4CZQ7	Q4DC97	TDY/TDY/TDY/ TDY/TDY	~.	
LmaMPK12	Q4Q7S2	LmxMPK12	DQ026026	TbMAPK4	Q585N3		Q4DHP2	I	TQY/TQY/TSY/THY	۰.	
LmaMPK13 (LF4)	Q4FVX2	LmxMPK13 (LF4)	DQ812905	MOK	Q38E60	I	Q4E4I5	Q4DWW0	TEY/TEY/ TEY/TEY TEY/TEY	Flagellar length (LMx)	[57]
LmaMPK14	Q4FYW2	LmxMPK14	DQ812906	I	Q57WV2	I	Q4D0S5	Q4D7J6	TDY/TDY/TDY/ TDY/TDY	Flagellar length (LMx)	[57]
LmaMPK15 ^a accn.; accessio	Q4Q3Y0 nn no.	LmxMPK15	DQ812907	I	Q389P3	1	Q4DKI1		TIY/TIY/TFY/TFY	۰.	

TABLE 2: Continued.

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of the proline-directed peptide binding pocket relative to the phosphorylation lip, perhaps placing these MAPKs in a separate subfamily.

KFR1 (KSS1- and FUS3-related kinase 1), the T. brucei homologue of L. mexicana LmxMPK1, mediates interferony-induced amastigote proliferation and phosphorylates serine residues on host histone H1, myelin basic protein, and β -casein [54, 55]. *T. brucei* TbECK1, which is the trypanosome homologue of L. mexicana LmxMPK6, possesses a carboxy-terminal extension that regulates kinase activity in all life cycle stages. Expression of a truncated TbECK1 protein lacking large parts of this extension caused T. brucei to grow slowly with abnormal morphology [61]. T. brucei procyclic forms lacking TbMAPK5, the homologue of L. mexicana LmxMPK5, likewise showed impaired differentiation into the bloodstream form [60]. TbMAPK2, the T. brucei homologue of LmxMPK4, regulates cell cycle progression from the procyclic (tsetse fly midgut) form to the bloodstream form [69]. TbMAPK5 controls T. brucei differentiation [60]. No functional studies of T. cruzi MAPKs have been published to date to our knowledge.

Phylogenetic analysis of *T. brucei* and *T. cruzi* suggests a single gene orthologous to the five putative MKK genes in *L. major* [51]. Only about one-third of the putative *L. major* MKKK genes have phylogenetic branching patterns consistent with the existence of orthologous genes in *T. brucei* and *T. cruzi* [51]. In most cases, *L. major* and *T. brucei* MKKK genes appear to be paralogues, having arisen from gene duplication events [51], suggesting significant evolutionary divergence in the circuitry of signaling cascades in Trypanosomatids. Three unique MKKKK genes have been identified in *T. cruzi* and two in *T. brucei* [51]. Functions have yet to be ascribed to any of these putative upstream MAPK activators.

3.2. Other Sarcomastigophora. Two MAPKs have been identified and characterized in the protozoan intestinal parasite *Giardia lamblia*, ERK1 and ERK2 (Table 1, Figure 1), each of which plays distinct roles in encystation [49]. In addition, one MAPK gene has been identified in the *Trichomonas vaginalis* genome [70]. However, functional studies have yet to be performed on MAPKs from either parasite.

3.3. Subphylum Sarcodina (the Amoebae). This subphylum of amoebas contains three human pathogenic genera: *Entamoeba*, *Naegleria*, and Acanthamoeba. The *E. histolytica EhMAPK* gene encodes a putative MAPK with significant homology to human ERK8 [71]. We are not aware of any further MAPK analyses in this genus or of any reports of MAPK genes or function in *Naegleria* or *Acanthamoeba*.

4. Protozoan MAPKs as Therapeutic Targets

MAPKs direct many functions critical to pathogen homeostasis and survival, including proliferation [62], differentiation [52, 53], regulation of cytoskeletal features such as the biosynthesis of flagella [56, 57, 63], and stress-responses [50]. Because protozoan MAPKs share many common structural features and are vastly more closely related to each other than to human MAPKs [18, 72], it should be possible to design drugs specifically or preferentially targeting protozoan MAPKs. For example, *Leishmania mexicana* LmxMPK1 and LmxMPK2 are essential MAPKs required for differentiation [52, 53], with corresponding homologues in other *Leishmania* species and in *T. brucei* and *T. cruzi* [51], but bearing scant resemblance to human MAPKs, making them excellent candidates for drug development [72]. Specifically targeting these MAPKs could have far reaching therapeutic potential since one drug could be used to treat a broad range of Trypanosomatid infections based on the high degree of homology between Trypanosomatid MAPKs [51].

P. falciparum Pfmap-2 is likewise an excellent druggable target as this MAPK is essential for the parasite to complete asexual replication in infected human erythrocytes [48] and it is highly dissimilar to human MAPKs. Although we have yet to determine which of the *T. gondii* MAPKs are essential to parasite survival, reducing BARKY expression dramatically impairs parasite virulence (Brumlik et al., submitted), making BARKY a useful target for MAPK inhibitor drugs.

Agents interfering with the function of MAPKs that affect stage differentiation, such as *T. gondii* BARKY, or affect parasite growth, such as *L. major* LmaMPK7 or *T. brucei* TbECK1, likely would be useful antiparasitic agents. *T. brucei* KFR1 is an interesting MAPK target, as it regulates effects of the host immune response (interferon- γ -induced amastigote proliferation) and could be considered in combination with an immune strategy. *L. mexicana* LmxMPK1 is homologous to KFR1 and could mediate similar effects, being a useful drug discovery target in this respect. Agents impairing the function of MAPKs controlling flagellar development or function, such as LmxMPK3, LmxMPK9, LmxMPK13, or LmxMPK14, could inhibit parasite dissemination and might be useful alone, or in combination with parasiticidal agents.

Our work with *T. gondii* BARKY demonstrates multiple MAPK splice variants that can occur naturally in parasites. A better understanding of the function of these splice variants could help develop agents specifically targeting variants relevant to disease pathogenesis. Likewise, our genomic analyses, and those of others, have demonstrated unusual repeat motifs in several protozoan parasite MAPKs (including in *T. gondii* and *Plasmodium* species) encoding large numbers of potential phosphorylation sites. An understanding of the functional significance of these motifs could help develop useful antiparasitic agents. Given the relatively unique nature of the phosphorylation site repeat motifs, these sites possibly could lead to highly parasite-specific drugs.

Protozoan MAPKs need not subserve critical functions to be useful drug discovery targets. For example, *L. mexicana* LmxMPK6 affects parasite morphology (which has indirect consequences on its growth rate following infection) and has homologues in related disease-causing Trypanosomatids. Drugs impairing LmxMPK6 function could be used in conjugation with existing anti-*Leishmania* therapies to boost their efficacy and could have broad-spectrum effects. Upstream components of the MAPK cascades such as the MKKs or MKKKs in pathogenic protozoan parasites are also potentially useful drug discovery targets. For example, the *L. mexicana* MKK, LmxPK4, controls parasite differentiation and thus is an excellent candidate. Because protozoan MKKs and MKKKs are even more distantly related to mammalian counterparts than MAPKs, a further potential advantage to this approach is that drugs inhibiting parasite MKK function could be less likely to have undesirable side-effects compared to drugs targeting specific MAPKs.

A potential disadvantage to targeting upstream MAPK regulators relates to our incomplete understanding of how they function. For example, MAPKs such as human p38 α are capable of MKK-independent activation and can undergo autophosphorylation in the presence of transforming growth factor- β -activated protein kinase 1 [73]. In this case, it would not be possible to block p38 α activation by targeting the conventional upstream MKKK and MKK components of the p38 MAPK cascade.

Many protozoan MAPKs possess vestiges of the common docking (CD) domain and ED site (Figure 1)—surfaceexposed acidic residues in human p38 α MAPK that facilitate binding to upstream and downstream MAPK partners [74, 75]. D³¹³, D³¹⁵, and D³¹⁶ comprise the CD domain in human p38 α MAPK. This region acts in concert with the ED¹⁶¹ site to bind to short strings of 2–5 basic amino acids situated on proteins with which p38 α interacts [74]. Protozoan MAPKs lacking a conserved CD domain (e.g., *Leishmania major* LMaMPK9 and 15) and/or ED site (e.g., *Leishmania major* LMaMPK3, 7, 9, 11, 15, and *T. gondii* TgMAPK1) are prime candidates for drug development since these domains have diverged considerably from their corresponding mammalian counterparts.

In addition, the highly variable carboxy-terminal extensions, which are present in over half the protozoan MAPKs shown in Figure 1, are excellent targets for drug development owing to their unique structures. Drugs targeted to these extensions would have a low probability of affecting mammalian MAPKs.

SB203580 is a pyridinylimidazole competitive ATP inhibitor affecting human p38 MAPK phosphotransferase activity through hydrogen bonding between its pyridine ring nitrogen and the MAPK backbone amide of \underline{M}^{109} in the THLM¹⁰⁹ motif (subdomain V; Figure 1) [39]. A second critical hydrogen bond occurs between a nitrogen atom on the imidazole ring and the invariant lysine in the VAX<u>K</u>⁵³ motif (subdomain II). Finally, the fluorophenyl ring of SB203580 interacts with the hydrophobic environment created by T¹⁰⁶ and H¹⁰⁷[39].

Because SB203580 is much smaller than ATP (as are all pyridinylimidazole p38 MAPK inhibitors), it does not fully occupy this region, leaving two large hydrophobic pockets on either side of the pyridine ring [39]. By designing novel pyridinylimidazoles or structurally related pharmacophores that properly fill the ATP binding pocket of pertinent protozoan MAPKs, it could be possible to develop novel antiparasitic agents that are more potent and specific than existing drugs. Such drugs will be less likely to have unintended consequences on host p38 MAPK, which is a potential drawback of several existing p38 MAPK inhibitors.

Recent molecular modeling studies using competitive ATP inhibitors against LCRK3 in *L. donovani*, a cyclindependent kinase that is a distant relative of the MAPK superfamily, indicate that such compounds could have significant inhibitory activity against *L. donovani* LCRK3 [76]. Our work has shown that the human p38 MAPK inhibitors RWJ67657, RWJ68198, and SB203580 reduced the replication of *L. donovani* promastigotes in axenic culture. Moreover, SB203580 effectively inhibited the replication of the bloodstream stage cultured *ex vivo* (Brumlik et al., unpublished observations).

X-ray crystallographic studies of human p38a MAPK complexed with ATP have demonstrated that the THLM¹⁰⁹ motif in the center of subdomain V (Figure 1) forms two critical hydrogen bonds with the adenosine moiety [77]. Based on our ClustalW alignment, many other amino acids can evidently serve this same purpose in other MAPKs (Figure 1; subdomain V), although the binding affinity of ATP (and competitive ATP inhibitor drugs) could be affected by such differences. Structural studies have further shown that the invariant GXGXXGXV38 motif in subdomain I coordinates the nontransferable α - and β -phosphates of ATP, while catalytic transfer of the y-phosphate is mediated by hydrogen bonding between an essential lysine in the VAXK53 motif (subdomain II), the RE⁶⁸ motif in subdomain III, and the underlined residues in the $HRD^{168}XK^{170}PXN^{173}$ motif (subdomain VIb) [78]. Thus, to design novel competitive ATP inhibitors against protozoan MAPKs, one must not only account for the invariant residues comprising the ATP binding site in all MAPKs but also pay particular attention to the permissible structural changes in subdomain V of protozoan MAPKs that specifically affect the binding of competitive ATP inhibitors.

We have shown that SB203580 [50] and another pyridinylimidazole human p38 MAPK inhibitor, RWJ67657, significantly inhibit BARKY autophosphorylation (Brumlik et al., unpublished observations). These agents reduced *T. gondii* proliferation *in vitro* [41] and treated otherwise fatal *T. gondii* infection in mice [42]. We further assessed the efficacy of two human p38 MAPK inhibitors to treat parasitic infections and showed that RWJ67657 and the pyrroloben-zimidazole RWJ68198 effectively blocked the replication of *P. falciparum* cultured in human erythrocytes *ex vivo*. Drug treatment resulted in trophozoites that were markedly diminished in size (Brumlik et al., submitted).

We demonstrated that RWJ67657 protected mice from otherwise fatal infection with the protozoan *Encephalitozoon cuniculi* [42] although it encodes no known MAPKs. Inhibition of host p38 MAPK could improve the host immune response to *E. cuniculi*, as has been demonstrated for *T. gondii* [79], or RWJ67657 could have therapeutic off-target effects in either the host or parasite. Better understandings of the mechanism of action in this case will further help drug development.

A large number of p38 MAPK inhibitors have recently progressed into phase I and II clinical trials, thus providing basic inhibitor pharmacophores that can be modified to target critical protozoan MAPKs specifically while at the same time having less host toxicity (a problem with many agents in the pyridinylimidazole class).

5. Conclusions

MAPKs play essential roles in virtually all eukaryotes. Thus, inhibiting protozoan MAPK functions represents a scientifically sound approach to developing novel classes of antiprotozoan agents. As protozoan MAPKs are only distantly related to mammalian MAPKs and have distinct active sites, it is reasonable to expect that selective agents can be developed to target pathogen proteins with minimal collateral effects on human counterparts.

Although only a very modest body of work on the structure and function of protozoan MAPKs currently exists, the available evidence already suggests the general utility of inhibiting protozoan parasite MAPK function as a treatment strategy. Several specific MAPK candidates have also already emerged from such work. As interest in MAPKs increases, the rate of important discoveries and their preclinical and clinical translation will also increase.

Specific roles for MAPKs cannot be predicted based solely on sequence similarity to protein homologues. For example, P. falciparum MAPK Pfmap-2 is essential for the completion of asexual replication in human erythrocytes [48] and yet its closest homologue in P. berghei, Pbmap-2 (with 93% amino acid sequence identity within its catalytic domains to Pfmap-2 [80]), is dispensable for both asexual replication and gametocyte formation in the mouse erythrocyte. Pbmap-2 instead plays a critical role in exflagellation in the mosquito midgut [81]. Thus, while the structure of the MAPKs themselves remains highly evolutionarily constrained even among closely related Plasmodium species, the circuitry of the various signal transduction pathways themselves has undergone significant divergent evolution. Therefore it is critical to establish specific roles of particular MAPKs prior to drug development.

Once the function of a MAPK from a pathogenic protozoan parasite has been established, one can exploit the phylogenetic differences between MAPKs of protozoan and metazoan origin to design specific MAPK inhibitors. Refining the structure of human MAPK inhibitor pharmacophores already in existence should speed development of new MAPK-inhibiting antiprotozoan drugs. We also expect to see additional new classes of drugs developed, which will be aided by additional structure/function studies. Targeting upstream MAPK regulators is an approach that also bears investigation, but which will likely lag owing to significant current knowledge gaps in understanding these regulators.

A considerable challenge is to persevere with such research given the relatively scant resources available for such work, in spite of the fact that over one-half billion people in many of the poorest parts of the world are infected by pathogenic protozoan parasites [82].

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Review Article Role of RNA-Binding Proteins in MAPK Signal Transduction Pathway

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Mitogen-activated protein kinases (MAPKs), which are found in all eukaryotes, are signal transducing enzymes playing a central role in diverse biological processes, such as cell proliferation, sexual differentiation, and apoptosis. The MAPK signaling pathway plays a key role in the regulation of gene expression through the phosphorylation of transcription factors. Recent studies have identified several RNA-binding proteins (RBPs) as regulators of MAPK signaling because these RBPs bind to the mRNAs encoding the components of the MAPK pathway and regulate the stability of their transcripts. Moreover, RBPs also serve as targets of MAPKs because MAPK phosphorylate and regulate the ability of RBPs to bind and stabilize target mRNAs, thus controlling various cellular functions. In this review, we present evidence for the significance of the MAPK signaling in the regulation of RBPs and their target mRNAs, which provides additional information about the regulatory mechanism underlying gene expression. We further present evidence for the clinical importance of the posttranscriptional regulation of mRNA stability and its implications for drug discovery.

1. Introduction

Cells need to respond to a variety of signals including extracellular stimuli, environmental stresses, and developmental signals as well as intrinsic information, to properly regulate gene expression and thereby execute biological functions. Given the multiplicity and complexity of the regulation of gene expression, failure to coordinate these regulatory mechanisms can contribute to the onset and progression of diseases such as cancer. Gene transcription and its regulatory mechanisms, together with recent genome-wide approaches for gene transcription analysis, have attracted the most attention with regard to gene regulation. However, it has become clear that additional stages in the gene expression cascade, including posttranscriptional events such as the control of mRNA degradation, stability, location, and translation, are equally crucial and require sophisticated regulation by various intracellular signaling pathways [1]. RNA-binding proteins have been shown to control the expression of numerous proteins by binding to the respective mRNA species encoding

proto-oncogenes, growth factors, cytokines, transcription factors, and other proteins in various cell types [2]. In particular, the phosphoregulation of RNA-binding proteins by MAPKs and/or kinases downstream of MAPKs, which can control the degradation or translation of target mRNAs, has attracted increasing attention. One of the most wellstudied model systems for the study of MAPK regulation is the fission yeast Schizosaccharomyces pombe (S. pombe). S. pombe, with its powerful genetics, is an excellent model system for the study of the mechanisms of signaling pathways in higher eukaryotes. In S. pombe, homologs of many signaling molecules involved in tumorigenesis, such as Ras, Rho, Protein kinase C, and MAPK, as well as drug targets, including TOR (target of Rapamycin) and the phosphatase calcineurin (target of the immunosuppressant drug FK506), are highly conserved. Spc1/Sty1/Phh1 and Pmk1 in S. pombe are homologs of mammalian p38 and ERK, respectively. The purpose of this review is to highlight the significance of several data using the fission yeast model system as well as other studies in higher eukaryotes that uncovered the cross-talk mechanism between MAPKs and RNA-binding proteins (RBPs) in the regulation of posttranscriptional gene expression, biological phenomena, and signal transduction [3].

2. RNA-Binding Proteins as Regulators of MAPK Signaling

Sugiura et al. developed a genetic approach to identify regulators of MAPK signaling on the basis of the antagonistic relationship between calcineurin phosphatase and MAPK Pmk1 in the regulation of Cl⁻ homeostasis in fission yeast (Figure 1) [4]. By utilizing this genetic interaction, Sugiura et al. identified several negative regulators of MAPK signaling, including *pmp1*⁺, *pek1*⁺, and *rnc1*⁺ (Figure 1). Pmk1 MAPK is a homologue of ERK/MAPK in mammals and regulates cell morphology, cytokinesis, ion sensitivity, and cell integrity in fission yeast [5]. The first gene identified encodes a dualspecificity MAPK phosphatase Pmp1 that dephosphorylates Pmk1 MAPK, thereby inhibiting Pmk1 signaling (Figure 1) [4]. The second gene identified was $pek1^+$, encoding an MAPK kinase (MAPKK), which phosphorylates and activates Pmk1 in its phosphorylated form, thus acting as an upstream MAPKK for Pmk1 (Figure 1) [6]. Surprisingly, Pek1 in its unphosphorylated state binds phosphorylated Pmk1, thereby inhibiting Pmk1 MAPK signaling. Hence, Pek1 acts as a phosphorylation-dependent molecular switch. The third identified gene encodes a novel KH-type RBP (Figure 1), which shares significant amino acid similarity with human Nova-2, human FMR-1, and Caenorhabditis elegans GLD-1. The third gene was designated as rnc1+ (RNA-binding protein that suppresses calcineurin deletion 1) because overexpression of the *rnc1*⁺gene suppressed Cl⁻ hypersensitivity of calcineurin-knockout cells (Figure 1).

2.1. MAPK Phosphatase mRNA as a Target of RNA-Binding Proteins. Rnc1 turned out to be a negative regulator of Pmk1 MAPK since Pmk1 MAPK was heavily phosphorylated in Rnc1-knockout cells but was barely phosphorylated in cells overproducing Rnc1. Based on the hypothesis that Rnc1 affects the metabolism of specific cellular mRNAs involved in the regulation of the Pmk1 signaling, the effects of Rnc1 on the mRNA stability of the MAPK phosphatase Pmp1 were examined, because Pmp1 was identified in the same genetic screen. The hypothesis is correct since Rnc1 binds and stabilizes an otherwise unstable Pmp1 mRNA, thus providing a novel functional link between MAPK signaling and an RBP through the mRNA stabilization of MAPK phosphatase (Figure 2) [7]. These findings are particularly important since Rnc1 directly affects the expression of MAPK phosphatase, thereby controlling the strength and duration of MAPK signaling pathway.

Pmp1 is highly similar to members of the dual-specificity MAPK phosphatase MKP-1 (MAPK phosphatase or DUSP1; dual-specificity phosphatase1), which plays a critical role in the negative regulation of MAPK signaling by dephosphorylating and inactivating ERK MAPK, JNK, and p38 [8–12]. Notably, Rnc1 recognizes and binds to the UCAU repeats in the 3'-UTR (untranslated region) of the Pmp1 mRNA (Figure 2) [7]. Mutations in the UCAU sequences greatly destabilize Pmp1 mRNA owing to the lack of binding of Rnc1 to Pmp1 mRNA [7]. Interestingly, the Nova-1 KH-type RNA-binding protein, which has been implicated in the pathogenesis of paraneoplastic opsoclonus-myoclonus ataxia (POMA), a disorder associated with breast cancer and motor dysfunction, binds to an element containing repeats of the tetranucleotide UCAU [8]. It would be intriguing to speculate that the mRNA stability of human MAPK phosphatase might also be regulated by an RNA-binding protein with or without KH-motifs.

Cell exposure to cytokines, growth factors, cell stresses, or activated oncogenes leads to the activation of MAPK and to the upregulation of mRNAs encoding a subset of MAPK phosphatase genes [9–18]. This induction of MAPK phosphatase expression has been extensively documented, and it is considered to involve transcriptional induction by various transcription factors, such as CREB and AP-1, via both MAPK-dependent and MAPK-independent pathways [19]. However, as shown in the study of Pmp1 in fission veast, MAPK phosphatase gene expression is regulated not only by its transcription rate but also by its rate of mRNA degradation in higher eukaryotes. Wong et al. reported that heat shock increases MKP-1 gene expression at both the transcriptional and posttranscriptional levels and the latter involves increased mRNA stability of MKP-1 [20]. Moreover, It has been shown that DUSP1/MKP-1 mRNA is stabilized by oxidative stress-induced binding of RBPs HuR and NF90 [21, 22]. In addition, HuR promotes MKP-1 mRNA recruitment to translation machinery and its translation efficiency.

Recently, Lin et al., Emmons et al., and Bros et al. suggested the importance of TTP, a zinc finger-AU-rich elements (AREs) RBP, in the regulation of MKP-1 mRNA [23-25]. TTP has been shown to bind to AREs located in the 3'-UTR of unstable mRNAs and to direct its target mRNAs for degradation. By utilizing RIP-Chip analysis in human dendritic cells, Emmons et al. identified 393 messages as putative TTP targets, 33 of which contained AREs, including dual specificity phosphatase1 (DUSP1/MKP1) mRNA. They further confirmed that the wild-type TTP significantly inhibits DUSP1/MKP1 expression by utilizing luciferase-DUSP 3'-UTR reporter, which contains repeats of the AUUUA sequences. Bros et al. revealed the role of TTP in the degradation of MKP-1 mRNA. They showed elevated expression of TTP target mRNAs, including MKP-1, in TTP^{-/-} dendritic cells. Lin et al. also showed that biotinylated MKP-1 AREs could bring down HuR and TTP in preadipocytes. Therefore, MKP1 mRNA is a target of several RBPs including HuR and TTP, and these RBPs are involved in the modulation of MKP-levels and MAPK activity through mRNA (de)stabilization of MAPK phosphatase, thus indicating that MKP-1 expression is tightly regulated transcriptionally and posttranscriptionally in many organisms, which ensures a rigid feedback control of MAPK signaling.

2.2. Feedback Regulation of MAPK Signaling by an RNA-Binding Protein. Interestingly, Rnc1 protein in fission yeast is regulated by MAPK phosphorylation. There are six putative



FIGURE 1: Molecular genetic approach to identify regulators of MAPK signaling using fission yeast model system. Upper panel: Calcineurin phosphatase and Pmk1 MAPK pathway play antagonistic roles in Cl^- homeostasis of fission yeast. Molecular genetic approach utilizing this genetic interaction identified regulators of Pmk1 MAPK. Lower panel: Calcineurin deletion cells (CN KO) failed to grow in the presence of 0.15 M MgCl₂. Overexpression of multicopy suppressor genes, including *pmp1*⁺ (MAPK phosphatase), *pek1*⁺ (MAPK kinase), and *rnc1*⁺ (KH-type RNA-binding protein) suppressed the Cl^- sensitivity of the calcineurin deletion cells.

MAPK-phosphorylation sites in Rnc1 protein and Pmk1 MAPK directly phosphorylates Rnc1 at T50 *in vivo* and *in vitro* [7]. Notably, this phosphorylation by MAPK regulates the RNA-binding activity of Rnc1, that is, the unphoshorylatable T50A mutant Rnc1 failed to bind Pmp1 mRNA while the phospho-mimic T50E mutation enhanced the RNA-binding activity of Rnc1 [7]. Therefore, when Pmk1 signaling is activated, Pmk1 MAPK phosphorylates Rnc1 and the phosphorylated Rnc1 protein more potently binds and stabilizes Pmp1 mRNA, resulting in upregulation of Pmp1 MAPK phosphatase, followed by the inactivation of Rnc1 as a target of the MAPK signaling cascade has revealed a novel feedback mechanism for the posttranscriptional regulation of MAPK phosphatase gene expression [7].

Similar negative-feedback mechanisms of the MAPK signaling pathway through posttranscriptional mRNA stabilization of MAPK phosphatase have been reported in other organisms. As described above, Casals-Casas et al. reported posttranscriptional mRNA stabilization of MKP-1 in macrophages and showed that this stabilization mechanism was partly dependent on p38 MAPK [19]. Recently, Bermudez et al. described the posttranscriptional regulation of DUSP6/MKP-3 phosphatase by MERK/ERK signaling and hypoxia [26]. They showed that the MEK/ERK pathway stabilizes dusp6 mRNA levels via its 3'-UTR. Moreover, hypoxia, a hallmark of tumor growth, increases dusp6 mRNA stability in a MEK activity-dependent manner. Interestingly, 2 RNA-binding proteins, TTP, and PUM2, a homolog of Drosophila pumilio protein, destabilize dup6 mRNA via its 3'-UTR. Therefore, although there is a diversity of the MAPK pathways concerned between S. pombe and higher eukaryotes, these studies suggest a conserved mechanism of feedback-regulation mechanism of MAPK signaling through

the mRNA stabilization of MAPK phosphatase. Thus, it is intriguing to speculate that some RBPs responsible for the mRNA stability of the MAPK phosphatase gene(s) might be regulated by p38 or ERK MAPK in higher eukaryotes. Attractive candidates for such RBPs may include TTP, since the well-known link between MK2 and TTP provides a plausible explanation for posttranscriptional regulation of MKP-1 by the p38 MAPK pathway.

3. RNA-Binding Proteins as Targets of MAPK Regulation

The MAPK pathway is known to play an important role in regulating gene expression, and this regulation has been primarily attributed to the activation of transcription factors via MAPK-dependent phosphorylation. However, since the time p38 MAPK was found to play a major role in the regulation of mRNA stability [27], accumulating evidence suggests that the stability of mRNAs can be controlled by RBPs under the regulation of various MAPK signaling pathways in different cellular systems [28–31]. These include tristetraprolin (TTP) [32–34], nucleolin [35], hnRNP-K [36, 37], hnRNPA0 [38], HuD [39], and the poly(A)-binding protein PABP1 [40], as well as Rnc1, Nrd1, and Csx1 in fission yeast [7, 41, 42].

Since the activation of p38 MAPK and its downstream MAPK-activated protein kinase-2 (MAPKAPK-2 or MK2) signaling pathway leads to the stabilization of AU-rich elements (AREs)-containing transcripts including various inflammation genes, ARE-binding proteins are likely targets for the p38 MAPK/MK2 pathway [43]. One of the well-characterized RBPs is TTP, which acts as a substrate of p38 MAPK/MK2. TTP binds to the ARE motifs in the 3'-UTR of


FIGURE 2: Negative feedback regulation of MAPK signaling mediated by an RNA-binding protein Rnc1. Rnc1 binds to the UCAU repeats located in the 3'-UTR of the Pmp1 mRNA. The activated Pmk1 phosphorylates Rnc1, leading to the enhanced activity of Rnc1, thereby binding to and stabilizing an otherwise unstable Pmp1 mRNA. Upregulated Pmp1 dephosphorylates and inactivates Pmk1, thus creating a negative-feedback loop that regulates the MAPK signalling.

several cytokine mRNAs including TNFα mRNA and targets them for degradation, thereby suppressing inflammation [33]. TTP^{-/-} mice developed severe inflammatory symptoms, including cachexia, spontaneous arthritis, dermatitis, and neutrophilia, mainly because of the overproduction of TNF α resulting from the increased stability of the TNF mRNA and subsequent higher rates of secretion of the cytokine [44]. Notably, macrophages derived from TTP^{-/-} MK2^{-/-} double knockout mice showed highly increased amounts of TNF almost identical to those in TTP-deficient mice, whereas in MK2-null mice, the TNF level was dramatically reduced in MK2-deficient animals compared to the wild type [45]. This genetic evidence strongly suggests that TTP is the main downstream element of MK2 in the posttranscriptional regulation of TNF in vivo. MK2 directly phosphorylates TTP at 2 serine residues (Ser52 and Ser178 in mouse TTP) in vitro and in vivo (Figure 3) [46]. Furthermore, TTP can be directly phosphorylated by p38 (Figure 3) [32].

Notably, phosphorylation by MK2 inhibits TTP activity. Several studies have described the molecular mechanisms underlying this regulation, although this subject is controversial. The phosphorylation of TTP by MK2 facilitates the dissociation of TTP from the ARE motif, thereby allowing the biosynthesis of TNF α [45]. Previous studies have also shown that MK2 is required for the maintenance of normal TTP levels and phosphorylation by MK2 leads to stabilization of TTP and inhibition of its mRNA destabilization activity. Notably, the phosphorylation of TTP at 2 serine residues (Ser52 and Ser178) allows binding of 14-3-3 adaptor proteins (Figure 3) [47, 48]. Furthermore, Stoecklin et al. showed that MK2-dependent phosphorylation of TTP releases it from stress granules and inhibits degradation of mRNAs containing AREs (Figure 3) [48]. Interestingly, recent studies



FIGURE 3: Control of ARE-containing mRNA degradation by TTP. Unphosphorylated TTP binds to the ARE and promotes degradation of the mRNAs. MK2 phosphorylates TTP at two serine residues, and this phosphorylation allows binding of 14-3-3 adaptor proteins and stabilization of the ARE-containing target mRNAs, such as TNF α . TTP can also be phosphorylated by p38. Phopshorylation also excludes TTP from stress granules.



FIGURE 4: The regulation of mRNA in MAPK signaling. MAP kinase signaling pathways regulate both transcriptional and posttranscriptional gene expression in various organisms. MAPK-signaling mediated phosphorylation regulates transcription factors and/or RNA-binding proteins, which resulted in mRNA regulation. Precise regulatory mechanism(s) of these proteins by MAPK signaling are not fully understood.

by Marchese et al. and Clement et al. described a molecular mechanism for the regulation of TTP function by MK2, involving the inhibition of recruitment of a deadenylase complex [49, 50]. In addition to TTP, MK2 also phosphorylates poly(A)-binding protein [40] and hnRNPA0 [51], thereby regulating their mRNA-binding capacity. Moreover, p38 MAPK phosphorylates the KH-type splicing regulatory protein (KSRP) and inhibits its binding and destabilization of mRNAs coding for proteins involved in the differentiation of myoblasts into myotubes, thus indicating that p38 regulates mRNA turnover by targeting KSRP [52].

Interestingly, the budding yeast p38 homolog Hog1 was shown to be essential for stabilization of these AREcontaining mRNAs [53], thus indicating that MAPKs also regulate mRNA stability in lower eukaryotes. In fission yeast, there is an interesting association between p38 and Csx1, an RBP crucial for survival following oxidative stress in S. pombe [42]. Csx1 is an RRM-type RBP with significant amino acid similarity to TIA1 in higher eukaryotes. Csx1 is phosphorylated upon oxidative stress in a Styl-dependent manner. Csx1 associates with and stabilizes atf1+ mRNA, which encodes a member of the ATF/CRE-binding protein family of transcription factors, including ATF2, in higher eukaryotes [42]. Moreover, Csx1 controls expression of the majority of the genes regulated by Spc1 p38 and Atf1 in response to oxidative stress. Notably, HuR RBP in higher eukaryotes stabilizes ATF-2 mRNA by binding to the 3'-UTR of ATF-2 mRNA in response to polyamine concentrations [54]. Consistently, HuR overexpression and depletion modulates ATF-2 mRNA abundance and resistance to apoptosis. These studies reveal a highly conserved mechanism of controlling MAPK-regulated transcription factors as well as several physiological responses, including oxidative stress and apoptosis through RBP.

4. Other Instances of MAPK-Mediated Phosphoregulation of RBPs: Cell Cycle Control Mediated by Modulation of mRNA Stability

Recent paper by Satoh et al. revealed an interesting link between RBP and cell cycle control mediated by modulating myosin mRNA stability [41]. They identified Nrd1, a highly conserved RNA-recognition motif (RRM)-type RBP, as a regulator of Cdc4, an essential myosin light chain, a key regulator of cytokinesis in fission yeast [55, 56]. Nrd1 binds to Cdc4 mRNA in vivo and in vitro, thereby stabilizing Cdc4 mRNA and regulating cytokinesis in fission yeast. Nrd1 shows significant sequence similarity with TIA-1 and the TIA-1-related (TIAR) proteins in higher eukaryotes [57, 58]. It has been reported that both TIA-1 and TIAR bind to RNA, with the preferred binding sequence being U-rich including the UCUU motifs present in the mRNAs [59, 60]. Notably, the mutation in the two UCUU sequences (cdc4M1M2) within the Cdc4 ORF abrogated the binding between Nrd1 and Cdc4 mRNA, and the cdc4M1M2 mutant cells exhibited cell separation defects, suggesting that the loss of Nrd1 binding affects cytokinesis.

Interestingly, Nrd1 is also phosphorylated by Pmk1 MAPK at two threonine residues (T40 and T126) and this phosphorylation negatively regulates Nrd1 activity to bind and stabilize Cdc4 mRNA and cytokinesis. Nrd1^{DD}, the phosphorylation mimic version of Nrd1, only weakly binds to Cdc4 mRNA, while Nrd1^{AA}, the unphosphorylatable version of Nrd1, in which T40 and T126 are substituted by alanine residues, strongly binds to Cdc4 mRNA [41]. The deletion of Pmk1 MAPK, similar to Nrd1 overexpression, stabilizes Cdc4 mRNA, thus providing the first example of MAPK-mediated cell cycle control through mRNA stabilization of myosin via RBP.

There are also interesting examples of cell-cycle control by modulation of mRNA stability in higher eukaryotes, especially with regard to DNA damage checkpoints [61]. Reinhardt et al. reported the involvement of MK2 activity in the regulation of Gadd45 α mRNA stability [38] and showed that the p38/MK2-dependent phosphorylation of 3 key targets involved in RNA regulation-hnRNP0, TIAR, and PARN-stabilizes an otherwise unstable Gadd45 α mRNA through its 3'-UTR, resulting in increased Gadd45 α protein levels. Gadd45 α itself is then required to maintain MK2 activity, which is critical for prolonged Cdc25 inhibition and maintenance of a G₂ arrest [38]. Thus, the positive feedback loop involving p38/MK2/Gadd45 α is essential to allow cells to recover from DNA damage, before committing to the next mitotic cell division. These reports on RBP-mediated cell-cycle control through mRNA stability provide further evidence for the phosphoregulation of RBPs through MAPK, thereby revealing an additional level of cell cycle regulation.

5. Clinical Importance of the Posttranscriptional Regulation of mRNA Stability and Its Implications for Drug Discovery

Considerable number of genes that code for AU-rich mRNAs, including cytokines, chemokines, growth factors, RBPs, and transcription factors, are commonly involved in both chronic inflammation and cancer [62]. Therefore, stabilization of the ARE-mRNAs via RBP can cause prolonged responses that subsequently may lead to chronic inflammatory diseases, such as rheumatoid arthritis and cancer. Indeed, neutralizing antibodies to $TNF\alpha$, one of the most important proinflammatory cytokine containing AREs, prevent arthritis in transgenic mice expressing ARElacking stable TNF α transcripts, which produce pathological overexpression of TNF α [63, 64]. Thus, abrogation of posttranscriptional pathways that mediate the expression of these proinflammatory genes may serve as a clinical target of inflammation. Furthermore, since the expression of MAPK phosphatase is closely linked to inflammation as well as malignancies of various cancer cells, targeting the mRNA expression of MKPs could represent a promising therapeutic target and elucidation of these mechanisms would be highly important for understanding the mechanism of cancer and inflammation. Since abnormal activation of the Ras/Raf/MAPK pathway can lead to cancer and since the p38/MK2 pathway plays a crucial role in inflammation, these MAPKs, together with the RBPs that regulate MAPKs, are attractive targets for new therapies for both cancer and/or inflammatory diseases such as Crohn's disease or rheumatoid arthritis. A large body of evidence from preclinical studies indicate a crucial role of p38 MAPK signaling in inflammation, and drugs that target p38 or MK2 and those that inhibit the development of inflammatory arthritis in mouse models are being investigated for the treatment of patients with rheumatoid arthritis and inflammatory bowel disease [65–67].

6. Concluding Remarks

We have presented several examples of functional relationships and cross talk regulatory mechanisms between RBPs and MAPK signaling in fission yeast model system and have drawn some parallels with animal cells. A recent paper by Belle et al. on the global investigation of protein halflives in budding yeast indicates that the protein products of genes that are coregulated on the transcriptional level also tend to be coregulated on the level of protein turnover [68]. Similarly, it would be intriguing to see the correlation between transcriptional and posttranscriptional regulation, since ERK MAPKs and the p38/MK2 axis regulate both the transcriptional and posttranscriptional gene expression of various immediate early genes involved in inflammation and stress responses [69]. Further studies are required to clarify the complex interplay between these transcriptional and posttranscriptional events during inflammation and cancer, and its regulation by RBPs and various signaling pathways including MAPKs pathways and others (Figure 4). Moreover, it would be important to elucidate the basis for the cooperative action of p38 MAPK and ERK pathways for optimal ARE-mRNA stabilization [70]. To clarify these mechanisms, the use of a simple eukaryotic model organism such as fission yeast could contribute to the field, since many RBPs and signaling pathways, including MAPKs and the major pathways of mRNA degradation and transcription, are highly conserved. Altogether, these data highlight an emerging role for RBPs as regulators of MAPK signaling and future targets for drug discovery.

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Review Article Erk in Kidney Diseases

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Acute or chronic kidney injury results from various insults and pathological conditions, and is accompanied by activation of compensatory repair mechanisms. Both insults and repair mechanisms are initiated by circulating factors, whose cellular effects are mediated by activation selective signal transduction pathways. Two main signal transduction pathways are activated during these processes, the phosphatidylinositol 3' kinase (PI-3K)/mammalian target of rapamycin (mTOR) and the mitogen-activated protein kinase (MAPK) cascades. This review will focus on the latter, and more specifically on the role of extracellular signal-regulated kinase (ERK) cascade in kidney injury and repair.

1. Introduction

In acute kidney injury (AKI) and chronic kidney disease (CKD), the kidney initiates activation of signaling pathways that act as intracellular communication lines that contribute to structural and functional manifestations. Among the wide array of signaling networks activated in the kidney, those containing mammalian target of rapamycin and mitogenactivated protein kinases (MAPKs) are more commonly studied. The role of mTOR in kidney disease has been extensively reviewed recently [1, 2]. We will focus on mitogenactivated protein kinases (MAPK), and, more precisely, on Erk, one of the MAPK, in this paper.

There are four different MAPK pathways in mammalian cells: extracellular signal-regulated kinase-1 and -2 (Erk1/2), c-Jun N-terminal kinase (JNK), p38MAPK, and extracellular signal-regulated kinase-5 (Erk5/BMK1) [3, 4]. Erk is mainly activated by mitogenic stimuli such as growth factors and hormones, and JNK and p38 are mostly activated by stress stimuli, and, are, therefore, sometimes categorized as stress kinases. Erk5 is activated by both stress stimuli and growth factors [4]. MAPKs are activated as part of three-tiered kinase cascades: they are activated by simultaneous phosphorylation on threonine and tyrosine residues by

dual-specificity MAP kinase kinases (MAPKK), which are themselves activated by serine/threonine phosphorylation by MAP kinase kinase kinases (MAPKKK) [3, 4] (Figure 1). Upstream of MAPKKKs lie additional protein kinases (such as Ste20-related protein kinases) or members of the Ras and Rho families of small GTPases. An additional layer of regulation has been described in proximal tubular epithelial cells in culture, in which activation of Src by PLC*y* lies upstream of Ras and activates Erk [5]. Pathways distinct from the kinase cascades described above can contribute to MAPK activities, and to cell specificity of MAPK activation. This paper will focus only on the role of the Erk1/2 pathway in kidney disease.

There are greater chances of restoration of renal morphology and function after acute kidney injury (AKI) [6] than in the case of chronic kidney disease (CKD); in the latter, similar repair mechanisms may be activated although they rarely lead to complete restoration. In response to acute or chronic stress, renal cells mount a response designed to limit the extent of injury which involves activation of antiproliferative and proapoptotic genes [6]. Later, this is followed by steps aimed at repairing the injury caused by the stress and the initial response; this reparative stage involves growth factors and proliferative as well as antiapoptotic signals [6]. In AKI, these repair mechanisms often lead to restoration of renal morphology and function, but in CKD sustained activation of repair mechanisms leads to aberrant cell proliferation, cell hypertrophy, and increased extracellular matrix deposition leading to progressive renal injury.

2. Compensatory Renal Hypertrophy: A Physiologic Adaptation

Immediately following removal of the contralateral kidney, hyperfiltration occurs in the remaining kidney, and is followed by compensatory growth, which is due to hypertrophy of mostly tubular epithelial cells [7]. This is a physiological response to the removal of contralateral kidney. After unilateral nephrectomy, mitogenic growth factors as well as TGF β are upregulated in the remaining kidney. Mitogenic factors trigger the differentiated epithelial cells to exit the G0 phase and enter the cell cycle [8]. This is caused by activation of cyclin D1 and D3-activated kinases, CDK4 and CDK6 [9]. Entry into the cell cycle initiates a synthetic program that allows the cells to accumulate enough material to reach a size that permits division into two daughter cells [10, 11]. However, the concomitant increase in TGF β stimulates the expression of cyclin-kinase inhibitors, such p27^{kip1} and p57kip2 in tubular epithelial cells [12]. This, in turn, prevents activation of cyclin E-CDK2 which is necessary to pass the restriction point and enter S phase, when DNA is replicated [13]. As a consequence, tubular epithelial cells are blocked in the late G1 phase of the cell cycle when protein synthesis and accumulation of newly synthesized materials, including proteins, occur leading to cell hypertophy.

As previously described, Erk plays a crucial role in signaling by mitogenic growth factors, it is likely that Erk is important in the first phase of the hypertrophic program, when epithelial cells enter the cell cycle. Furthermore, Erk mediates upregulation of TGF β in tubular epithelial cells [14]. Thus, by promoting two crucial events in this process, entry into the cell cycle and upregulation of TGF β that prevents DNA replication, Erk plays a fundamental role in the development of compensatory kidney growth after unilateral nephrectomy.

3. Acute Kidney Injury

3.1. Ischemia/Reperfusion. Ischemia/reperfusion (I/R) injury induces both functional and morphological changes in the kidney. Necrosis, predominantly of the proximal tubule, is the hallmark of this model of renal injury. After ischemic injury, both the Erk and phosphatidylinositol 3 kinase (PI3K) signaling pathways are activated in the kidney [15, 16], notably in the region where thick ascending limbs predominate [15], whereas stress-activated kinases, p38MAPK and JNK are activated in tubular epithelial cells [15]. Erk activation is due to oxidant-induced activation of a EGF Receptor/Ras/Raf signaling cascade [16] and blockade of Erk reduces cell survival after I/R injury [15]. In addition, the renoprotective effect of preconditioning, using short period





of ischemia [17] or cyclosporine A or FK506 [18] prior to an I/R insult appears to depend on decreased activation of p38MAPK and JNK, and increased activation of Erk. Similarly, inhibition of monoamine oxidase after an I/R insult potentiates Erk activation and increases proliferation but decreases necrosis of tubular cells [19]. However, a protective role for Erk was called into question by Alderliesten et al. who showed that in vivo inhibition of Erk significantly reduced renal damage after I/R injury [20].

3.2. Cisplatin-Induced Nephrotoxicity. Cisplatin is one of the most effective chemotherapeutic agents used for the treatment of malignant tumors, but its use is limited by its side effects, including nephrotoxicity, neurotoxicity, ototoxicity, hair loss, nausea, and vomiting [21]. Nephrotoxicity is the major dose-limiting factor during cisplatin treatment, as approximately one-third of patients experience AKI within days after cisplatin treatment [22]. Injury and death of renal tubular cells are the key pathological occurrences in cisplatin nephrotoxicity [23, 24], and Erk seems to play an important role in this process.

In tubular epithelial cells in culture, cisplatin stimulation of Erk is mediated by an EGF-R/Src cascade [25]. Activated Erk accumulates in mitochondria following cisplatin treatment and impairs its function contributing to apoptosis; and inhibition of Erk with U0126 ameliorates mitochondrial dysfunction and apoptosis of tubular epithelial cells [26]. In mice, injection of U0126 decreases Erk activation following cisplatin administration, and offers significant renoprotection, accompanied by decreased inflammation markers, caspase 3 activity and apoptosis [27]. These data show that Erk activation mediates the renal inflammation and tubular epithelial cell apoptosis in cisplatin-induced nephrotoxicity.

4. Chronic Kidney Injury

4.1. Polycystic Kidney Disease. Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common human monogenic diseases, with an incidence of 1:400 to 1:1000 [28, 29]. It is characterized by the development and gradual enlargement of multiple fluid-filled cysts within both kidneys. These cysts encroach upon and destroy normal adjacent nephrons [28]. Cyst growth and higher kidney volumes correlate with diminishing clearance function of the kidney in ADPKD [30]. Abnormalities of tubular cells lining the cysts in ADPKD include increased proliferation, increased apoptosis, abnormalities of protein sorting and polarity, and disorganization of the underlying extracellular matrix [31, 32]. In DBA2-pcy/pcy mice with polycystic kidney disease, robust Erk activation is detected in the cyst epithelium;administration of an inhibitor of the Erk pathway, PD184352, effectively reduces Erk activation and inhibits cyst-induced gain in kidney weight, cyst index and improves renal function [33]. This study underlines the important role of Erk in the formation of cysts that results from aberrant proliferation of the tubular epithelium. It also identified Erk as a potential therapeutic target in ADPKD. Since targeting mTOR with rapamycin or everolimus did not significantly ameliorate ADPKD in human subjects [34, 35], the identification of novel therapeutic targets such as Erk could be of interest.

4.2. Chronic Mesangioproliferative Glomerulonephritis-Induced by Anti-Thy1 Antibody. Anti-Thy1 experimental nephritis is a well-established model of experimental mesangioproliferative glomerulonephritis in the rat. Anti-Thy1 antibody binds specifically to mesangial cells and triggers complement-induced mesangiolysis, followed by rebound proliferation of mesangial cells [36]. In this model, maximum proliferation of mesangial cells is observed 6 days after injection of anti-Thy1 antibody, and it is accompanied by a significant activation of Erk and inactivation of p38MAPK in the glomerulus [37]. Treatment of rats with heparin reduces glomerular cell proliferation as well as Erk activation and restores p38MAPK activation [37]. Injection of U0126, the MEK1 inhibitor, to rats 3 days after injection of Thy1 blocks Erk activation and returns the number of proliferating glomerular cells to normal at day 6 [38]. Together, these studies demonstrate that Erk mediates and p38MAPK opposes the proliferative response in mesangioproliferative glomerulonephritis.

The role of ERK in cellular proliferation has been extensively studied. In resting conditions, Erk is anchored in the cytoplasm by its association with the microtubule network [39] and other scaffolding proteins, such as Sef [40] and PEA15 [41]. Activation of Erk by mitogens is biphasic: a first, robust, and transient phase peaks at 5-10 min and is followed by a second, weaker but more sustained phase lasting several hours [42, 43]. Nuclear translocation of Erk occurs within minutes of stimulation, is reversible upon removal of the mitogenic stimulus, and lasts throughout the G1 phase of the cell cycle [44]. Nuclear Erk is inactivated during the G1/S phase transition and is exported back to the cytosol [44]. In the nucleus, Erk phosphorylates and activates transcription factors, such as Elk1 and c-Fos, which stimulate the expression of several growth-related genes [45]. It is important to remember that Erk activation in the nucleus is

required but not sufficient for successful progression through the cell cycle [8].

4.3. Rat Model of Progressive Membranous Nephropathy (Heymann Nephritis, PHN). Heymann nephritis is a model of membranous nephropathy characterized by complement-dependent injury to podocytes. Injection of sublytic doses of complement (C5b-9) causes kidney damage in rats, that is restricted to podocytes. In these cells, C5b-9 causes DNA damage and cytoskeleton remodeling, along with Erk activation and upregulation of p53 and p21^{cip1} [46]. Actin cytoskeleton remodeling seems to cause localized activation of Erk and selective phosphorylation of substrates, such as cPLA2 but not Elk1 [47].

Inhibiting Erk in vivo in PHN worsened DNA damage in podocytes and reduced the upregulation of p21^{cip1} [46], suggesting a protective role of Erk in this model. In spite of chronic activation of Erk after overexpression of MEK, its upstream kinase, exacerbates complementmediated podocytes in culture [47], suggesting a deleterious role for Erk. A possible explanation for this discrepancy is that overexpression of MEK causes excessive Erk activation that far exceeds what is seen in PHN in vivo and overcomes the protective role of Erk observed in vivo. These observations also emphasize the importance of context in assessing the role of Erk, while it may mediate injury response in the kidney in one context, for example, cisplatinum, it is involved in renal defense in another, for example, PHN.

4.4. Unilateral Ureteral Obstruction. Unilateral ureteral obstruction (UUO) in rodents generates progressive renal fibrosis due to marked renal hemodynamic and metabolic changes, followed by tubular injury and cell death by apoptosis or necrosis, with interstitial macrophage infiltration. Proliferation of interstitial fibroblasts with myofibroblast transformation leads to excess deposition of the extracellular matrix and renal fibrosis. Immediately following obstruction, a biphasic activation of Erk occurs: an early, transient phase (30 min after obstruction) of stimulation is seen in the collecting duct; this is followed by a sustained phase (4 to 7 days) in the collecting duct, the tubular epithelial cells and the cortical interstitium [48-50]. The latter phase of Erk activation has been attributed to oxidative stress [49], and its blockade prevents interstitial cell proliferation and interstitial macrophage accumulation, but not the activation of interstitial fibroblasts and renal fibrosis [50]. These results show that Erk plays a selective and limited role after UUO.

4.5. Diabetic Nephropathy. Characteristic morphologic changes in diabetic nephropathy (DN) include kidney hypertrophy, glomerular basement membrane thickening, and the accumulation of mesangial matrix [51, 52]. Later in the disease, progressive tubulointerstitial injury and fibrosis are observed [51, 52]. Renal enlargement, one of the first structural changes in DN, is due to the hypertrophy of existing glomerular and tubular cells rather than to cellular proliferation [51–54].

4.5.1. Erk and Global Protein Synthesis. As described earlier, cellular hypertrophy is the consequence of a failure to escape the late G1 phase, when global protein synthesis takes place, and to complete the cell cycle. Cellular accumulation of protein during hypertrophy could be due both to increase in its synthesis and decrease in degradation. Stimulation of protein synthesis is due to the coordinated increase in the transcription of their respective genes, and the translation of their mRNAs; the latter is thought to be the rate-limiting step in gene expression [55, 56]. Regulation of mRNA translation can occur at the levels of both increase in efficiency of translation and capacity for translation. The former involves events occurring in the initiation and elongation phases of mRNA translation [57], whereas the latter is regulated at the level of ribosome biogenesis and assembly.

(i) Erk in Initiation and Elongation Phases of Translation. When a signal for increasing protein synthesis is received, the cell ramps up the process of translating the codons in mRNA into respective peptide, that is, mRNA translation. Translation occurs in three phases [56, 58]. During the initiation phase, several eukaryotic initiation factors (eIFs) assemble into two large multimeric complexes, that is, the preinitiation complex (PIC) consisting of eIF1, 1A, eIF3, eIF5, eIF2+ initiator methionyl tRNA and the 40S ribosomal subunit, and, the eIF4F complex consisting of eIF4E, eIF4G, and eIF4A [59]. The cap-binding protein eIF4E is held inactive by its binding protein, 4E-BP1, in the resting state, and is released by phosphorylation of the latter when translation is stimulated [60]. Free eIF4E undergoes phosphorylation on Ser209 and forms eIF4F complex with eIF4G and eIF4A and binds to the cap of mRNA at its 5' end. Due to binding between eIF3 and eIF4G, a bridge is now formed between PIC and eIF4F, which brings 40S ribosomal subunit to the proximity of the mRNA. After a complex set of reactions, the 60S subunit joins 40S subunit forming the 80S ribosomal unit and the eIFs fall away from the complex but initiator methionyl tRNA remains. The 80S unit successfully localizes to the AUG codon on the mRNA, marking the end of initiation phase of translation.

All three of translation phases, initiation, elongation, and termination are exquisitely regulated [56, 57]. For instance, both initiation and elongation phases are regulated by the PI3K-Akt-mTOR signaling pathway, which ensures the coordinated activation of these two critically important events and the continuous "flow" of mRNA translation and ultimately protein synthesis. Additional layers of regulation allow fine tuning of mRNA translation. One such layer is represented by the Erk signaling pathway, which indirectly regulates the initiation phase of mRNA translation. One of Erk substrates, MAPK interacting kinase1 (Mnk1) phosphorylates eIF4E [61-63]. In contrast to mTOR-dependent phosphorylation of 4E-BP1 which is transient, Mnk1dependent phosphorylation of eIF4E is persistent [64]. In renal epithelial cells undergoing hypertrophy under the stimulation of VEGF, Ser209 phosphorylation of eIF4E appears to be needed for increase in protein synthesis [5]. Investigation of signaling regulation showed that VEGF recruited VEGF

receptor type 2 to activate phospholipase $C\gamma$, Src, Raf, MEK, Erk pathway in stimulating Mnk1, eIF4E phosphorylation, and protein synthesis (ibid). These data show that Erk plays an important role in increasing the efficiency of translation.

(ii) Erk and Ribosome Biogenesis. Cell growth, or increase in cell mass, requires a large increase in the number of ribosomes. In mammals, transcription of ribosomal DNA coding for ribosomal RNA is activated by upstream-binding factor (UBF) and selectivity factor 1. UBF activates rRNA gene transcription by recruiting RNA polymerase I to the rDNA promoter, by stabilizing binding of TIF-IB/SL1, and by displacing nonspecific DNA-binding proteins such as histone H1 [65, 66]. UBF function is regulated by phosphorylation by various kinases, such as Erk, casein kinase 2 (CK2), and cyclin-dependent kinases (CDK) [67]. Phosphorylation of Thr117 and Thr201 by Erk is essential for transcription elongation by RNA polymerase I [68, 69], whereas phosphorylation by CK2 and CDKs in the carboxy-terminal domain affect protein-protein interactions and activates rDNA transcription indirectly [70, 71]. Recent work from our lab has shown that high-glucose-induced hypertrophy and protein synthesis in glomerular epithelial cells is associated with increase in rDNA transcription (to generate ribosomal RNA) demonstrating ribosomal biogenesis. This process is dependent on UBF phosphorylation on Ser388 that was partly under the control of Erk [72]. Increase in Ser388 phosphorylation of UBF was also found in kidney parenchyma from rodent models of type 1 and type 2 diabetes, coinciding with kidney hypertrophy [72], suggesting that increased ribosomal biogenesis occurs in vivo in hypertrophic kidney during diabetes.

Ribosome assembly is an extremely complex process that involves four ribosomal RNAs (rRNAs) and approximately 80 ribosomal proteins [73]. In addition, more than 200 additional proteins and noncoding RNAs participate in the production of 60S and 40S ribosomal subunits. Ribosome assembly and activity requires posttranslational modifications of ribosomal proteins and Erk is involved in this process. In addition to generating ribosomal RNA, augmented protein synthesis involves activation of a number of proteins that are part of 40S (small, S) and 60S subunits (large, L). Ribosomal protein 6 (rpS6) and 3 (rpS3) of the 40S subunit are commonly studied.

(*iii*) *Ribosomal Protein S6 (rpS6)*. Ribosomal Protein S6 activation occurs during cell growth and it is a determinant of cell size [74]. Activation of rpS6 requires phosphorylation of conserved serine residues that is mediated by p70^{S6K} (S6K1) [75]. However, the fact that in mice lacking both S6K1 and S6K2, phosphorylation of rpS6 on Ser235/236 was conserved indirectly indicated that other kinases could compensate. Further studies have shown that this phosphorylation was mediated by p90^{rsk} that was itself activated by Erk [76]. Although Erk-driven rpS6 phosphorylation is functionally relevant in T-cell receptor signaling in CD8⁺ T cells [77], its significance in renal disease has not yet been established.

(*iv*) *Ribosomal Protein S3* (*rpS3*). Ribosomal Protein S3 possesses two independent functions. In the cytosol, it is part of the 40S subunit of the ribosome and as such participates in the initiation of mRNA translation [78]. In the nucleus, it functions as an endonuclease and is involved in DNA repair [79]. The subcellular localization of rpS3 is regulated by phosphorylation by several kinases, including Erk [80]. Phosphorylation of rpS3 on Ser42 by Erk triggers its nuclear translocation [80]. Activation of Erk can thus repress mRNA translation and stimulate DNA repair, preventing the cells from translating aberrant mRNAs. It is therefore possible that sustained activation of Erk during kidney hypertrophy in type 2 diabetes [81] could lead to a decreased availability of rpS3 for mRNA translation, thereby limiting protein synthesis and cell growth.

4.5.2. Erk and Selective Protein Synthesis. Accompanying renal hypertrophy, the accumulation of extracellular matrix proteins such as type IV collagen, laminin, fibronectin, is the other cardinal manifestation in diabetic kidney disease. Progressive accumulation of matrix proteins accounts for renal fibrosis in diabetic kidney disease and is a major determinant of progressive loss of kidney function [82]. The role of the Erk pathway on the stimulation of selective synthesis of matrix proteins was investigated by our group. We reproduced the type 2 diabetic milieu (high glucose and high insulin) and studied its effect on synthesis of an important kidney extracellular matrix protein, laminin β 1, by proximal tubular epithelial cells in culture. High glucose and high insulin, alone or in combination, triggered rapid synthesis of laminin β 1 within 5 min of stimulation [83]. All three conditions activated the PI3K-Akt-mTOR and Erk pathways in parallel and inhibition of either pathway prevented the rapid synthesis of laminin β 1. In insulin-treated kidney epithelial cells, Erk stimulation was downstream of PI3K, which may partly explain the common mode of regulation of laminin synthesis by both kinases [84].

5. Conclusion

Erk figures prominently in mediating kidney cell responses to a variety of diverse stimuli. This occurs in the physiologic setting such as compensatory kidney hypertrophy and in pathologic conditions such as models of glomerular and tubulointerstitial diseases. It should be noted that in the setting of diseases, it is not wise to generalize that Erk activation always results in tissue injury in the kidney. As reviewed above, inhibition of Erk could worsen specific kidney diseases. Thus, it is important to extend our knowledge of disease-specific regulation of Erk and then contemplate ways to modulate its activity. This requires better understanding of the role of Erk in all phases of individual kidney diseases before its modulation is planned.

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