REGULATED EXPRESSION ALLOWS HIGH LEVEL PRODUCTION AND SECRETION OF HIV-1 gp120 ENVELOPE GLYCOPROTEIN IN *DROSOPHILA* SCHNEIDER CELLS

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We have established a stable, continuous culture *Drosophila* Schneider 2 cell line that efficiently expresses a secreted, truncated form of the HIV envelope gp120 protein in a regulated manner. The *Drosophila* produced recombinant gp120 protein is highly glycosylated, is recognized by gp120-specific monoclonal antibodies, binds to the CD4 receptor and has the ability to inhibit syncytia formation between uninfected CD4$^+$ cells and HIV infected cells. We conclude that this recombinant *Drosophila* envelope protein is an appropriate mimic of the authentic viral envelope protein. Thus, the *Drosophila* cell provides a continuous, stable culture system for the efficient expression of secreted forms of complex surface glycoproteins in quantities sufficient for detailed analyses.

There has been increasing interest in obtaining recombinant forms of the proteins encoded by HIV viruses in sufficient quantities and in pure form for detailed biochemical, biophysical, and biological analyses. One HIV protein, which has been refractory to efficient recombinant expression, is the viral envelope (gp120/gp160) that binds to the cellular receptor (CD4) on T helper lymphocytes and facilitates viral entry into the cell. Fragments of the envelope protein have been successfully produced using bacterial systems[1–4], and a full-length, soluble protein has been secreted from *E. coli* although it does not bind CD4[5]. Expression in yeast has resulted in insoluble, full length protein that is inactive in CD4 binding and nonglycosylated[6,7]. Both mammalian[8–11] and insect[12–15] viral vectors have been used successfully to produce small amounts of recombinant HIV *env* protein that binds CD4. However, these lytic systems are difficult to operate at larger scale and do not afford the ease of production associated with stable, continuous cell culture systems. Recent data indicate that *Spodoptera*, the insect cell commonly used for baculovirus expression, can be used for the creation of stable cell lines by standard transfection procedures[16]. However, gene expression using this system was found to be relatively inefficient. To date, only one system has been reported that allows stable and continuous production of moderate levels of a fusion protein consisting of most of the gp120 coding region attached to the N-terminus of the herpes virus gD protein[17]. Although the product of this system exhibits many features of the HIV *env* protein, the typical mammalian cell systems require months of selection for high gene copy number to attain relatively efficient production levels.

We have previously described a *Drosophila* cell-based expression system that allows relatively rapid (~3 weeks) creation of a stable, continuous cell culture that efficiently expresses foreign gene products in a tightly regulated manner[18–20]. The system utilizes the *Drosophila* metallothionein promoter and hygromycin selection to achieve multicopy insertion of a stably integrated transcription unit in a single transfection/selection procedure. In this report, we apply this *Drosophila* system to the expression and secretion of a recombinant form of the gp120 envelope protein of the HIV-1 BH10 isolate. We demonstrate that efficient, regulated expression of a secreted form of gp120 can be obtained in stable, continuous insect cell culture. We structurally and functionally characterize this product and find that the recombinant protein is an accurate mimic of the authentic viral gp120.

RESULTS AND DISCUSSION

Stable, continuous culture of an integrated multicopy gp120 transcription unit. In mammalian cells, continuous, stable expression of an almost full length gp120 fusion protein has been achieved by truncation of the mature protein sequence at amino acid residue 31 and replacement of the signal sequence and 30 amino acids of the mature N-terminus with the signal sequence and N-terminal 25 amino acids of the herpes simplex glycoprotein[17]. We adapted a similar strategy by removing the envelope glycoprotein signal sequence and 31 amino acids of the mature protein and replacing it with the signal sequence and N-terminus of human tissue plasminogen activator[21] (tPA). The final expression vector (pMt120Δ32) contains the tPA signal sequence[22] and first 4 amino acids of the mature tPA protein joined in-frame to amino acid 32 of the HIV-1 BH10 mature envelope glycoprotein sequence[23,24]. To ensure that the protein would have the authentic C-terminus and would be secreted from the cells we truncated the gene by inserting a stop codon precisely at the normal processing site of gp120 and gp41[25] (just after amino acid 481 of the mature protein). Transcription was under the control of the *Drosophila* metallothionein promoter and signals for polyadenylation were derived from the SV40 early region. Correct cellular processing of the tPA signal sequence would result in a secreted envelope protein (gp120*) lacking the first 31 amino acids from the mature form of gp120 and containing 4 amino acids derived from tPA at its amino terminus.

The gp120 expression plasmid was introduced into *Drosophila* cells by cotransfection with a second vector

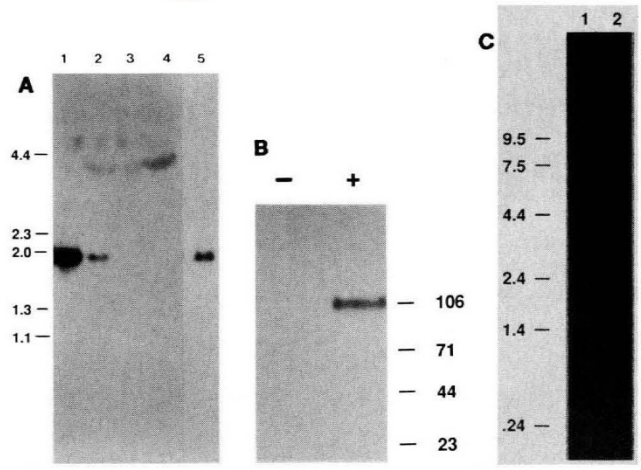

FIGURE 1 Analysis of the copy number and regulated expression of the *Drosophila* cell line containing the gp120 transcription unit. A: Determination of copy number using Southern analysis of the hydromycin-resistant cell line genomic DNA. Genomic DNA was digested with restriction enzymes that excise the gp120 gene from pMt120Δ32 on a 1.7-kb fragment. The DNA fragments were electrophoresed, transferred to nitrocellulose and hybridized with a [^{32}P]935 nucleotide probe, which only encodes the C-terminal half of gp120. Lanes 1–3, purified 1.7 kb gp120 fragment corresponding to 100, 10 and 1 gp120 gene copies per cell, respectively. Lane 4, untransformed control cells. Lane 5, hygromycin-resistant cell line. DNA standards are in kilobases. [This figure is a composite of lanes from the same film.] B: Western blot analysis of regulated gp120* expression. Conditioned medium from the hygromycin-resistant cell line was harvested after 5 days with or without metal induction. Unfractionated 20 μl samples were analyzed by Western blot using gp120-specific antiserum followed by ^{125}I-protein A. (–), conditioned medium from uninduced cells. (+), conditioned medium from induced cells. C: Northern analysis of regulated gp120* expression. RNA was extracted from hygromycin-resistant cells after cells after four days with or without induction. Samples were analyzed by Northern blotting with the same [^{32}P]gp120-specific probe used in Fig. 2A. Lane 1, RNA from uninduced cells. Lane 2, RNA from induced cells. RNA standards are in kilobases.

carrying the *E. coli* hygromycin B phosphotransferase gene, which confers resistance to hygromycin B[19]. A stable polyclonal cell line was generated after 3 weeks of hygromycin B selection. Chromosomal DNA was isolated and fragments were generated using restriction enzymes that excise the gp120 gene from the original pMt120Δ32 vector. Southern analysis of these fragments and comparison to purified DNA standards demonstrated that the selected cell population contained on the average about 20 copies of the gp120 transcription unit per cell (Fig. 1A). This copy number was maintained after serial passage of the cell line either in the presence or absence of hygromycin B selection for several months (20 passages) indicating that the sequences appeared to have been stably integrated into the host cell chromosomal DNA. A similar analysis using a hygromycin B-specific probe showed that the cell line also contained that starting vector (not shown). Hence, in a single transfection experiment we have obtained a stable, continuous cell system containing multiple copies of the gp120 transcription unit.

Regulated expression of HIV envelope. We examined the expression levels of the cell line described above by harvesting both culture medium and cells before and after metal induction. Gp120* production was analyzed by Western blotting on SDS-PAGE using a gp120-specific polyclonal rabbit antibody. The results (Fig. 1B) indicate that gp120* was selectively expressed in response to metal induction, and secreted into the culture medium. Moreover, the protein was of the size expected for a highly glycosylated derivative of the envelope protein encoded by our expression vector. In contrast, only low levels of gp120* were found associated with the cell pellet fractions. The titer of gp120* in the medium, as measured by ELISA, increased continually over 5 days and reached a level of 2 μg/ml at a cell density of 10^7 cells/ml. Furthermore, this cell line has been cultured in the absence of selection for at least 20 passages with little or no decrease in gp120* titer.

We also characterized the envelope mRNA levels before and after metal induction using Northern blotting analysis. The blots were probed with an envelope-specific probe containing only envelope sequence. The results (Fig. 1C) show that, as expected, a 1.7kb gp120 RNA rapidly accumulates in the transfected cell line in response to metal induction, compared with low to undetectable amounts of this RNA found in uninduced cells. This RNA induction is consistent with that observed at the protein level. A second shorter transcript of 1.4 kb is also detected (Fig. 1C) with the envelope-specific probe. This transcript could result from cryptic splicing, premature termination or RNA degradation of the envelope transcript. In any case, this smaller transcript cannot encode the entire gp120* and, hence, the level of its occurrence predicts a concomitant loss in potential gp120 expression.

Characterization of gp120*. Gp120* was purified from the conditioned media by a simple two step procedure involving ion exchange and immunoaffinity chromatography. SDS-PAGE analysis indicates that the final product is >95% pure (Fig. 2). The overall yield of the purified gp120* from conditioned medium was 75% as measured by ELISA. Gas-phase amino acid sequencing gave a single major amino-terminal sequence G-A-R-S-D-T-E-V-X-N-V-W-A (authentic envelope sequence beginning at +32 is underlined). This sequence is identical to that predicted from the DNA sequence and demonstrates that *Drosophila* recognizes the human tPA signal sequence and processes it correctly, thereby leaving four N-terminal amino acids from the mature human tPA gene product fused to amino acid +32 of the HIV *env* gene.

The HIV envelope protein is known to be highly glycosylated with a complex array of Asn-linked oligosaccaride structures[26–27]. The importance of these carbohydrate structures in virus recognition of the human CD4 receptor has been reported to be both critical[5,30–32] and insignificant[33–34]. Insect cells are known to glycosylate proteins and use the same recognition signals as do mammalian cells[35]. However, insect cells apparently lack the enzymes present in mammalian cells that are required to convert the core high mannose structure to the more complex glycan derivatives[35]. Hence, the carbohydrate composition of gp120* should be distinct from that found in the viral envelope protein.

An initial characterization of the carbohydrate composition of gp120* was performed using a Dionex carbohydrate analyzer[36]. The carbohydrate composition of gp120* was determined to be 249 moles of mannose, 58 moles of glucosamine, and 8 moles of fucose per mole of protein. The high percentage of mannose, the lack of galactose and sialic acid, and approximately two glucosamines for each of the 24 predicted glycosylation sites suggests the expected presence of the high mannose structures and some fucose-containing structures. This is consistent with the known carbohydrate compositions of other glycoproteins expressed in *Drosophila* and other insect cells[35] and distinct from the reported structures of viral[28,29] or mammalian recombinant gp120[26,27].

Functional analysis of gp120*. We examined several properties of gp120* relating to its function. These included: (1) The ability of the protein to be recognized by

a panel of monoclonal antibodies known to recognize different epitopes of the viral envelope; (2) its ability to recognize and bind to the human CD4 receptor; and (3) its ability to block virus-mediated cell fusion (i.e. syncytia formation) between infected cells and $CD4^+$ uninfected cells.

Five different monoclonal antibodies[37] were used to initially probe the structure of gp120*. Enzyme immunoassays[37] indicated that each monoclonal antibody recognized the recombinant protein to the same extent as a control gp120 protein produced in mammalian cells using a vaccina virus vector (Claudine Bruck and Clotilde Thiriart, unpublished results). The results suggest that the recombinant protein, although lacking 31 N-terminal residues and having a different carbohydrate composition, retains the general structure of the authentic viral protein.

Perhaps a more critical test of the recombinant protein is its ability to bind to the CD4 receptor, an interaction which is known to require appropriate folding of non-adjacent epitopes on gp120[38]. Mixtures of gp120* and a soluble recombinant form of the CD4 receptor[39] (sCD4) were immunoprecipitated using either an antibody to CD4 or an antibody to gp120 neither of which interfere with the gp120-sCD4 interaction. In both cases analysis indicated that the gp120/sCD4 complex was coprecipitated (data not shown). Using an excess of sCD4, it was shown that virtually all of gp120* could be precipitated indicating that essentially all the recombinant material was active. Most importantly, a direct comparison of sCD4 binding for gp120* versus viral gp120 (Fig. 3A) demonstrated equivalence of the two proteins.

Using these same reagents, we also examined the relative ability of gp120* (again compared to viral gp120) to interfere with virus-mediated syncytia formation. Uninfected CD4+ cells were incubated with cells chronically infected with either the HIV-1 BH10 or RF isolate in the presence or absence of gp120* and separately, viral gp120. The results (Fig. 3B) demonstrate that the gp120* was able to completely abolish syncytia formation in cells infected with either virus. Moreover, the relative ability of the gp120* to inhibit was essentially identical to that of the authentic viral material.

In conclusion, we have demonstrated that the *Drosophila* produced gp120 protein, gp120*, is an appropriate mimic of the viral envelope. The *Drosophila* system has the capability of rapid production of stable cell lines, which efficiently secrete this gene product in a regulated fashion. This has facilitated the production of sufficient quantities of protein for a variety of detailed analyses. Recently, we have expanded the use of the *Drosophila* system to the production, isolation and comparison of the envelope proteins from a variety of HIV-1, HIV-2 and SIV isolates (M. Ivey-Hoyle et al., Proc. Nat. Acad. Sci. USA, in press). We find that the system lends itself readily to continuous production of up to 30 mg of protein per liter of *Drosophila* cell culture (M. Ivey-Hoyle, unpublished). This system should be useful for expression and secretion of other complex secreted proteins.

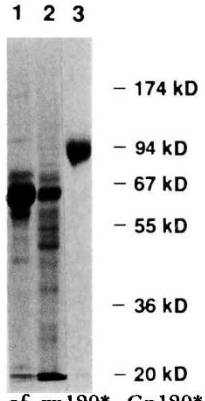

FIGURE 2 Purification of gp120*. Gp120* was purified by ion-exchange and immunoaffinity chromatography. Pooled fractions were analyzed by SDS-PAGE and Coomassie staining. Lane 1, conditioned medium. Lane 2, S-Sepharose pool. Lane 3, Immunoaffinity purified and concentrated gp120*. Protein molecular weight standards are in kiloDaltons (kD).

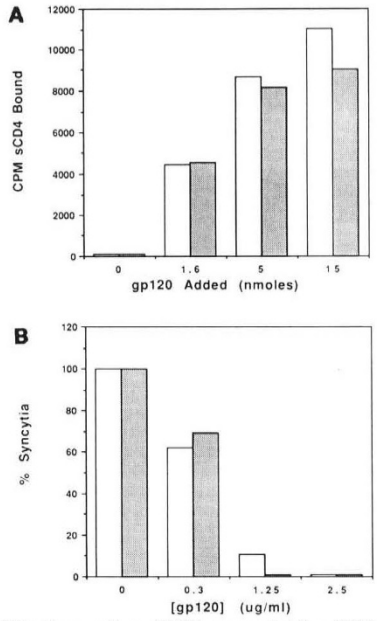

FIGURE 3A: Binding of gp120* to soluble CD4. A constant amount of ^{125}I-sCD4 was added to increasing amounts of gp120* or viral gp120. Bound sCD4 was pelleted with anti-gp120 178.1 antibody followed by protein A-Sepharose. Shaded and open bars represent viral gp120 and gp120*, respectively. B: Syncytia inhibition assay. Increasing concentrations of viral gp120 and gp120* was incubated with chronically infected CEM-HIV-1 (IIIB) cells and uninfected MOLT-4 cells. The number of syncytia were estimated by microscopic analysis. The number of syncytia in the absence of gp120 (56) was equated to 100% syncytia. Shaded and open bars represent viral gp120 and gp120*, respectively.

EXPERIMENTAL PROTOCOL

Construction of recombinant plasmids. pMt120Δ32 was derived from pMttPA which was constructed as follows. First, pML2[39], a pBR322 derivative, was digested with Sal1 and Aat2 and filled in. This fragment is ligated with a filled in EcoR1-Stu1 fragment from pDM131 containing the *Drosophila* metallothionein promoter[18] and a filled-in Hind3-Sac1 fragment from pDSP1[21] containing the entire coding region of tPA and the SV40 early polyadenylation signal. pMttPA was converted to pMt120Δ32 by first removing most of the human tPA gene (leaving the signal sequence and mature N-terminus) using a Bgl2 (mung bean)-Xba1 digestion and replacing it with an Nde1 (mung bean)-Sty1 fragment containing the HIV-1 III_B BH10 gp120 gene[23] (nucleotides 5986-7298). A Sty1-Xba1 synthetic linker was used that precisely encoded the authentic C-terminus of gp120 and introduced a stop codon directly after the last gp120 codon[25]. The final pMt120Δ32 vector contains the gp120 gene (missing the first 31 amino acid codons) fused in frame to the tPA N-terminus. Expression of the gene is controlled by the metallothionein promoter and polyadenylation signals are supplied by SV40 early.

Cell culture, transformation and induction. The *D. melanogaster* Schneider 2 cell line[40] was maintained in M3 medium[41] supplemented with 5-10% heat-inactivated fetal bovine serum. DNA transfection, hygromycin B selection using pCOdhygro and induction were as described earlier[18].

Northern and Southern analysis. Total cellular RNA and DNA was analyzed as earlier described[18]. For the Southern blot, the

gp120 transcription unit was excised from 1 μg of cellular DNA on 1.7-kb HindIII-SacI fragment prior to electrophoresis on a 1% agarose gel. Both blots were probed with an *env*-specific nick-translated 935 bp Stu1-Sac1 DNA fragment. This fragment extends from the middle of the gp120 coding region to the stop codon and, therefore, contains only envelope sequence.

Western analysis of gp120* induction. Conditioned medium from the hygromycin B-resistant cell line was harvested after 5 days in the presence or absence of copper induction at a cell density of 5×10^6/ml. Conditioned medium was electrophoresed on a 10% SDS-PAGE and analyzed by Western blotting techniques. The gp120 protein was visualized using a gp120-specific rabbit polyclonal antiserum followed by incubation with ^{125}I-labeled protein A (New England Nuclear).

Gp120 ELISA. Goat anti-mouse IgG (Southern Biotechnology) was absorbed onto a Nunc-Immuno 96-well plate at 4°C for 16 hours. 178.1 antibody[37] was added for 30 min. at 37°C followed by the gp120 samples for one hour, 37°C. Rabbit anti-gp120 antibody was added followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Cappel) each for one hour, 37°C. After each addition the plates were washed 3 times with 10 mM Na phosphate, pH 7.4, 0.15 mM NaCl (PBS). Protein reagents were diluted in PBS, 0.5% BSA, 0.05% Tween 20. Enzyme activity was assayed using *o*-phenylenediamine dihydrochloride as substrate (S. Mai et al., in preparation).

Analysis of gp120-CD4 binding. Mammalian cell-expressed sCD4 protein[42] was immunoprecipitated with 5-fold molar excesses of either monoclonal antibody OKT4A (Ortho Pharmaceutical) or gp120* followed by 178.1 anti-gp120 monoclonal antibody[37] and protein A-Sepharose (Pharmacia). Gp120* was immunoprecipitated with either monoclonal antibody 178.1 or sCD4 protein followed by OKT4 monoclonal antibody (Ortho Pharmaceutical) and protein A-Sepharose. The precipitates were detected by immunoblot analysis using rabbit polyclonal antiserum to either gp120 or sCD4[42]. For comparison of viral gp120[30] vs. gp120*, increasing amounts of gp120 was incubated with ^{125}I-sCD4 (M. Ivey-Hoyle et al., Proc. Natl. Acad. Sci, in press) for 42 hours at 4°C. Bound ^{125}I-sCD4 was separated from unbound protein by immunoprecipitating the sCD4/gp120 complex with 178.1 anti-gp120 for 1 hour at 4°C followed by protein A-Sepharose. Pelleted material was washed and counted. Background was 600 cpm. Each binding reaction was performed in triplicate and averaged.

Gp120* purification. *Drosophila* conditioned medium was made 70 kallikrein inhibitor units/ml in aprotinin, 5 mM in EDTA and 1 mM in PMSF and chilled to 4°C. Gp120* was captured on an S-Sepharose fast flow column (25.2 cm × 11 cm, Pharmacia) equilibrated in 50 mM MES, pH 6.0 and eluted in the same buffer containing 400 mM NaCl. The eluate was made 0.2 mM in PMSF, 1 μM each in leupeptin and pepstatin, and 0.1 mM in EDTA. The solution was loading on an immunoaffinity column (purified 178.1 mouse monoclonal antibody[37] immobilized on Affi-gel HZ [Bio Rad] according to manufacturer's instructions) equilibrated in 20 mM MES, pH 6.0. The column was washed with 20 mM MES, pH 6.0, 0.5% Tween 20, 1.0 M NaCl. Gp120* was eluted with 0.1 M acetic acid, pH 2.8 and fractions were immediately neutralized by addition of 1 M Tris, pH 10.4. Pooled fractions were diafiltered into PBS and concentrated to 1 mg/ml before storage at 70°C.

Amino acid, N-terminal sequence and carbohydrate analysis. Purified gp120* was hydrolyzed for 18 hours at 110°C and subsequently analyzed on a Beckman 6300 Amino Acid analyzer, N-terminal sequence was determined by loading purified gp120* sample directly into the gas-phase sequencer (Applied Biosystems) for analysis. The resulting Edman cycles were dried, dissolved in 15 μl of sample buffer and 10 μl of each analyzed by RP-HPLC. For carbohydrate composition analysis, 2 μg of gp120 were hydrolyzed in 1 ml of 2M TFA at 100°C for 5 hours and were lyophilized. Monosaccharides were determined by a carbohydrate analyzer (Dionex Corp) using the eluants and the detector conditions described earlier[36]. Monosaccharides were separated using 15 mM NaOH for 10 min. followed by a linear increase to 30 mM at 20 min.

Syncytia inhibition assay. Increasing concentrations of viral gp120 or gp120* were incubated with 5000 CEM cells chronically infected with HIV-1 (IIIB) and 7.5×10^4 uninfected MOLT-4 cells[30]. The cells were maintained in 96 A/2 well plates in a final volume of 100 μl of growth medium for 24 hours at 37°C. Duplicate wells were tested for each concentration. The number of syncytia was estimated by microscopic examination at a 40× magnification. The number of syncytia in the absence of gp120 (56) was equated to 100% syncytia.

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