Characterization of Stable Chinese Hamster Ovary Cells Expressing Wild-Type, Secreted, and Glycosylphosphatidylinositol-Anchored Human Immunodeficiency Virus Type 1 Envelope Glycoprotein

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We generated Chinese hamster ovary cell lines that stably express wild-type, secreted, and glycosylphosphatidylinositol (GPI)-anchored envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1). The cells expressing wild-type Env (WT cells) express both the precursor gp160 and the mature gp120/gp41 and readily form large syncytia when cocultivated with CD4⁺ human cells. The cells expressing secreted Env (SEC cells) release 140-kDa precursor and mature 120-kDa envelope glycoproteins into the supernatants. The cells expressing GPI-anchored Env (PI cells) express both 140-kDa precursor and mature gp120/gp41 envelope glycoproteins, which can be released from the cell surface by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). Both the secreted and PI-PLC-released envelope glycoproteins form oligomers that can be detected on nonreducing sodium dodecyl sulfate-polyacrylamide gels. In contrast to the WT cells, the SEC and PI cells do not form syncytia when cocultivated with CD4⁺ human cells. The availability of cells producing water-soluble oligomers of HIV-1 Env should facilitate studies of envelope glycoprotein structure and function. The WT cells, which readily induce syncytia with CD4⁺ cells, provide a convenient system for assessing potential fusion inhibitors and for studying the fusion mechanism of the HIV Env glycoprotein.

Human immunodeficiency virus type 1 (HIV-1) delivers its genome to host cells by fusing its envelope with cellular membranes (37, 45, 52, 55). This process is mediated by an envelope glycoprotein complex composed of two noncovalently associated subunits. The surface glycoprotein, gp120, binds the CD4 cellular receptor (8, 31, 34, 39), and the transmembrane glycoprotein, gp41, initiates membrane fusion (13, 15, 19, 46). The molecular details of how gp120/gp41 causes membrane fusion are not known but may involve conformational changes (1, 23, 42, 50) that expose the fusion peptide of gp41 in a manner similar to that of the well-characterized influenza virus hemagglutinin (4, 54, 60–62).

gp120 and gp41 are derived from the fusion-inactive precursor protein, gp160 (38), which has approximately 850 amino acids. gp160 is heavily glycosylated and cleaved intracellularly to yield the mature gp120/gp41 (33, 38). gp160 forms homooligomers in cells expressing the glycoprotein (10, 47, 51, 57), and gp120/gp41 is oligomeric on the surface of virions (51, 58).

In this study, we generated and characterized Chinese hamster ovary (CHO) cell lines that stably express wild-type, secreted, or glycosylphosphatidylinositol (GPI)-anchored Env glycoproteins (WT, SEC, or PI cells, respectively). These cell lines provide stable sources of membrane-anchored and soluble envelope oligomers. The first approach to prepare a soluble envelope glycoprotein ectodomain was to introduce a stop codon in the HIV-1 envelope just prior to the transmembrane domain. Because this strategy does not always produce an ectodomain that retains native oligomeric structure (53), we also engineered the HIV envelope glycoprotein such that it would be membrane anchored via a GPI tail. Oligomeric forms of the G protein from vesicular stomatitis virus (7), T-cell receptor (35), major histocompatibility complex class II alpha/

beta complex (59), acetylcholinesterase (9), influenza virus hemagglutinin (29), and Rous sarcoma virus envelope glycoprotein (18) have been generated in this manner. GPI anchors are susceptible to cleavage by the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) (12). Therefore, the GPI-anchored HIV-1 Env ectodomain should be released as an oligomer after treatment with PI-PLC. The WT, SEC, and PI cell lines would also allow us to compare the fusion potential of transmembrane-anchored, secreted, and GPI-anchored forms of the envelope glycoprotein. This comparison is important for understanding the fusion mechanism of the HIV-1 Env glycoprotein and fusion proteins in general.

MATERIALS AND METHODS

Recombinant DNA. Envelope sequences from the HXB-2 molecular clone of HIV-1 (*Sac* [5999]-*Xho* [8896] sites, as numbered in Human Retroviruses and AIDS data base of the National Institute of Allergy and Infectious Diseases) were taken from plasmid HXB-2env, kindly provided by Dan R. Littman (44). To facilitate further subcloning, sequences from this plasmid were mutated by using the oligonucleotide GCATCTCCGAGCTAGATAAG to recreate the destroyed *Xho* site at position 8896 that resulted when the HXB-2 *Sac-Xho* fragment was originally ligated into the *Sal* site in the pSM polylinker. The secreted mutant, pSM-HXB2sec, was created by site-directed mutagenesis using the synthetic oligonucleotide GCTGTGGTATATATAAGAATTCATAATGATAGTAGG to create a stop codon at nucleotide position 8270 and a new *EcoRI* site just downstream of the stop. Consequently, the transmembrane protein is truncated at isoleucine, the second residue before the putative transmembrane domain (Fig. 1) (20, 33). The GPI-anchored mutant, pSM-HXB2pi, was created by ligating to the *Xho* site of envelope a 1.5-kb nucleotide fragment containing positions 1018 to 2030 from

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FIG. 1. Diagram of wild-type (WT), secreted (SEC), and GPI-anchored envelope glycoproteins stably expressed in CHO cells. Truncation sites and predicted amino acids around the transmembrane region are shown. C-terminal gp41 ectodomain residues are in bold-face.

decay-accelerating factor (DAF) (40) that contains a signal for the replacement of a transmembrane domain with a GPI anchor (43). The synthetic oligonucleotide GGCTGTGGTATATAAACCAAATAAAGGAAGTGG was used to loop out the unwanted sequences of envelope and DAF, such that nucleotide 8272 from envelope was positioned next to nucleotide 1018 from DAF. With this construct, nine amino acids from DAF that are contiguous with the GPI anchor were added to the C terminus of the gp41 ectodomain (Fig. 1).

All constructs were sequenced over the mutated region to verify that no unintentional mutations were introduced during mutagenesis. Prior to generation of stable transfectants, the constructs were expressed transiently in COS-7 cells (American Type Culture Collection, Rockville, Md.) to verify that they encoded proteins of the correct molecular mass.

For stable expression, the envelope sequences were inserted into the EE14 expression vector (Celltech, Ltd., London, England), which has a glutamine synthetase minigene for selection and amplification and a human cytomegalovirus promoter. The envelope sequences from pSM-HXB2/Xho, pSM-HXB2sec, and pSM-HXB2pi were inserted into the *EcoRI* site in the EE14 polylinker.

Tissue culture. Using the calcium phosphate DNA coprecipitation method as specified by Celltech, CHO-K1 cells (American Type Culture Collection) were cotransfected with 10 μ g of the pEE14-envelope constructs and 7 μ g of an expression vector encoding HIV pREV (26), provided by

Tristram Parslow (University of California, San Francisco). After 36 h, the transfected cells were switched to selective medium containing the drug methionine sulfoximine (MSX; Sigma Chemical Co., St. Louis, Mo.). Selection was begun at 25 μ M MSX in glutamine-deficient minimal essential medium (GMEM-S) (29) and gradually increased to 300 μ M over the course of 5 weeks. Surviving colonies were picked, replated, and screened for envelope expression by Western blot (immunoblot) analysis. Approximately 3 months posttransfection, the concentration of MSX in the medium was increased further and maintained at 400 μ M. The cells described in this report represent the lines expressing the highest levels of envelope glycoprotein after selecting three successive colonies.

Antibodies and reagents. The rabbit polyclonal anti-gp120 antiserum (PB 32) was provided by Phillip W. Berman, Genentech, Inc. (South San Francisco, Calif.). The human monoclonal anti-gp41 antibody and T-cell lines HUT-78, CEM, SupT-1, and Molt4/clone 8 were provided by Jay A. Levy (University of California, San Francisco). Dan R. Littman (University of California, San Francisco) provided the HeLa-T4 and 3T3-T4 cell lines and soluble CD4. The anti-V3 loop mouse monoclonal antibody 110.3 was obtained from Genetics Systems (Seattle, Wash.). CD4-immunoglobulin G (IgG) (5) and purified gp120 produced in CHO cells were provided by Genentech, Inc. The phycoerythrin-conjugated anti-mouse monoclonal antibody was purchased from Caltag Co. (South San Francisco, Calif.).

Immunoprecipitations. Cells in 10-cm-diameter dishes were incubated in cysteine-methionine-deficient medium (Select-Amine; GIBCO BRL) for 90 min before incubation overnight in 5 ml of cysteine-methionine-deficient medium containing 2% supplemented calf serum and 0.25 mCi of [³⁵S]cysteine-methionine (ICN Biochemicals, Irvine, Calif.). Methods for immunoprecipitating have been described previously (28). Briefly, the cells were washed three times with phosphate-buffered saline (PBS), lysed with 1% Nonidet P-40–0.1 M Tris (pH 7.5) containing a protease inhibitor cocktail, immunoprecipitated, run on nonreducing sodium dodecyl sulfate (SDS)-polyacrylamide gels, enhanced by soaking in Amplify (Amersham) for 15 min, dried, and autoradiographed (Kodak XAR film).

CD4 binding. CD4 binding assays were performed as immunoprecipitations in which 250 ng of CD4-IgG per 1-ml sample of cell lysate was used as the primary antibody.

Surface expression. Cells were removed from dishes by incubation with PBS containing 0.5 mM EDTA and 0.5 mM EGTA [ethylene glycol-bis-(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid]. Approximately 10⁶ cells were pelleted, resuspended in 100 μ l of FACS (fluorescence-activated cell sorting) buffer (2% supplemented calf serum in PBS without cations), and incubated with a 1:500 dilution of anti-gp120 mouse monoclonal antibody 110.3 on ice for 30 min. The cells were then washed once with FACS buffer, incubated with a 1:20 dilution of phycoerythrin-conjugated goat anti-mouse monoclonal antibody on ice for 30 min, then washed again, and immediately analyzed on a FACSScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

Release of GPI-anchored envelope glycoprotein. Adherent cells in 10-cm-diameter dishes were washed twice with PBS without cations before addition of 8 μ l of PI-PLC (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 1.2 ml of incubation buffer (1 mg of bovine serum albumin and protease inhibitors per ml in PBS without cations). The cells with enzyme and incubation buffer were then incubated for 1 h at 37°C with intermittent rocking. Cells were then washed once

with PBS and immediately stained and analyzed by flow cytometry as described above. Samples of the PI-PLC supernatants were also harvested, cleared of cell debris by centrifugation at 14,000 rpm for 12 min, and analyzed for envelope glycoprotein by immunoprecipitation with CD4-IgG or antibodies as described above.

Syncytium assay. Approximately 5×10^5 CD4⁺ cells and envelope-expressing cells, removed from stock dishes by incubation in calcium- and magnesium-free PBS containing 0.5 mM EDTA and 0.5 mM EGTA, were coplated in six-well dishes containing 3 ml of GMEM-S medium. The cocultures were incubated overnight at 37°C, and syncytia were visualized microscopically the next day. For photography, supernatants were aspirated, and cells were rinsed with PBS, fixed with 10% formaldehyde-PBS for 3 min, quenched with one wash of 100 mM glycine-PBS, and stained with Giemsa dye. Kodak ASA 400 Tri-X pan film and an orange filter were used for photography.

RESULTS AND DISCUSSION

Transfections. CHO cells were transfected with HIV-1 envelope constructs (Fig. 1), and cultures were selected with MSX and screened for envelope glycoprotein expression by Western blot analysis. In two separate transfections, it was relatively easy to identify colonies in the SEC and PI cultures that were positive for Env expression (9 of 12 for both cell types). Conversely, only 2 of 21 colonies analyzed from the WT cultures expressed detectable levels of Env. Expression of all three forms of the envelope glycoprotein has been stable in the selected cell lines, even after more than a year of continuous culture.

The fact that fewer colonies expressed wild-type Env relative to the secreted or GPI-anchored ectodomain is consistent with previous reports that the carboxy terminus of gp160 modulates export, processing, and stability of the envelope glycoprotein in some cell types (2, 14, 21). The relative ease of obtaining GPI cells suggests that membrane association of Env does not, in itself, limit envelope expression.

Expression. Cell lysates of the WT cells showed the 160-kDa precursor glycoprotein and the mature gp120. Cell lysates of the SEC and PI cells showed uncleaved precursors, which ran as approximately 140-kDa proteins, as well as mature gp120 (data not shown). The migration of the precursors in the SEC and PI cells is consistent with the deletion of 173 amino acids from gp41.

The gp120 of all three cell lines was readily detected in media of overnight cultures (Fig. 2). The appearance of significant quantities of gp120 from the WT and PI cells probably reflects the tendency of the gp120 from this strain of HIV-1 to shed from gp41 (17, 48). The amount of shed or secreted envelope glycoprotein from overnight cultures was estimated by Western blot analysis, by comparison with known quantities of purified gp120 produced in CHO cells. With this method, we estimated that the WT, SEC, and PI cells released, respectively, 10, 60, and 20 ng of gp120 per ml in overnight supernatants from $\sim 5 \times 10^6$ cells (data not shown).

On low-percentage, nonreducing SDS-polyacrylamide gels, the SEC supernatants showed bands of apparent molecular masses of ~ 260 and ~ 370 kDa, in addition to the 140-kDa precursor and 120-kDa bands (Fig. 2). The ~ 260 - and ~ 370 -kDa bands probably represent oligomers of the uncleaved gp140 precursor and/or the gp120/truncated gp41 complex. The oligomers were readily immunoprecipitated by both CD4-IgG (Fig. 3) and anti-gp120 polyclonal antisera (data not shown). Of note, our system to generate a secreted envelope

FIG. 2. Immunoprecipitation of supernatants from ³⁵S-labeled cells with CD4-IgG. Radiolabeled proteins were analyzed on a nonreducing SDS-4% polyacrylamide gel. Storage of samples at -20°C reduced the efficiency of oligomer detection. The faint high-molecular-weight band seen in the PI lane appears to be nonspecific, as it can be seen in control lanes when exposed to match the background seen in the PI lane. EE, control cells. Positions of commercial molecular weight standards (cross-linked phosphorylase b; Sigma) are shown on the right in kilodaltons.

glycoprotein differs from previous ones (3, 10, 22) in the exact placement of the stop codon, the use of wild-type sequences around the gp120/gp41 cleavage site (3), and the use of a different expression vector and cells.

Cell surface expression. The presence of gp120 in the supernatants of all cell lines suggested that the envelope glycoproteins are exported to the cell surface. To obtain more direct evidence for this, we analyzed live cells by flow cytometry (Fig. 3A). Using a monoclonal antibody against the V3 loop (antibody 110.3), we saw significant fluorescence in all three cell lines, with the highest cell surface staining in the PI cells. Surface staining of the SEC cells may be due to nonspecific sticking of the secreted envelope glycoprotein to the cell surface. Surface staining of a similarly truncated envelope glycoprotein expressed with a vaccinia virus system has previously been reported (11). A biotinylation analysis revealed that the WT and PI cells both express precursor and mature glycoproteins on their surface (data not shown).

Release of GPI-anchored envelope glycoprotein. When the PI cells were treated with PI-PLC, approximately half of the surface envelope glycoprotein was released from the cell surface, indicated by a 50% reduction in surface staining in flow cytometry experiments (Fig. 3C). Since the WT cells (Fig. 3B) did not show decreased gp120 surface staining on flow cytometry after PI-PLC treatment (Fig. 3B), it is unlikely that the PI-PLC treatment resulted in degradation or nonspecific cleavage of the surface glycoproteins. GPI-anchored proteins frequently have inositol ring modifications that provide additional sites for membrane anchoring that render them resistant to treatment with PI-PLC (12). This could explain why some of the envelope glycoproteins on the GPI cells remained associated with the cell surface after treatment with PI-PLC.

After treatment with PI-PLC, the supernatants were analyzed on nonreducing, SDS-4% polyacrylamide gels. The PI cells consistently released the 140-kDa precursor and mature gp120 as well as a band of ~ 260 kDa. In some gels, a weak band of ~ 370 kDa was also seen (Fig. 4, lane 4). The ~ 260 -

FIG. 3. (A) Relative fluorescence intensity by flow cytometry of cells stained with a monoclonal antibody against the V3 loop of gp120. (B) WT cells stained before and after PI-PLC treatment. (C) PI cells stained before and after PI-PLC treatment.

and ~370-kDa bands line up with the oligomer bands seen when SEC cell supernatants were run on nonreducing gels (Fig. 4, lane 5). Poorer detection of the ~370-kDa band in the PI sample may be due to lower protein loading, relative instability of the PI oligomer in SDS, and/or the production of different oligomeric forms in the PI versus SEC cells. It will be important to compare the oligomers from the SEC and PI cells with those produced from other constructs designed to yield oligomeric ectodomain fragments of the HIV envelope glycoprotein (3, 11, 22). The gp120 seen in the supernatants of PI-PLC-treated WT cells (Fig. 4, lane 2) and mock-treated PI cells (Fig. 4, lane 3) probably represents residual shed gp120 that was not completely washed from the cell surface prior to PI-PLC treatment or continued gp120 shedding during the PI-PLC incubation.

Syncytia. The most convincing demonstration of the production of functional gp120/gp41 at the surface of WT cells was the appearance of numerous large syncytia when the WT cells

FIG. 4. Env proteins in the supernatants of cells treated with PI-PLC. Cells were radiolabeled, and Env proteins in the culture supernatants were immunoprecipitated with a mixture of anti-gp41 monoclonal and anti-gp120 polyclonal antisera and analyzed on a nonreducing SDS-4% polyacrylamide gel. (The combination of both antibodies gave stronger signals than did either antibody alone.) Lanes: 1 to 4, washed cells treated with mock (-) or PI-PLC (+) buffer; 5, overnight supernatant from SEC cells. A >400-kDa band appears to be nonspecific, as it is present in the control (lane 1). The appearance of gp120 in lanes 2 and 3 is explained in text.

were cocultured with CD4⁺ human cells (Fig. 5B). The syncytia induced by the WT cells mimicked those produced by HIV-1-infected cells. The WT cells formed syncytia with all CD4⁺ human cells tested, including HeLa-T4 (Fig. 5B), HUT-78, CEM, Supt-1, and Molt4/clone 8 (data not shown), but not with mouse 3T3-T4 cells (data not shown). Soluble CD4 and the anti-V3 loop antibody 110.3 inhibited syncytium formation by at least 95% (data not shown). Neither the SEC nor PI cells formed syncytia with CD4⁺ cells (Fig. 5C and D). We did not expect the SEC cells to form syncytia, because the secreted Env lacks a membrane anchor, and all known fusion proteins contain a hydrophobic transmembrane domain in addition to a hydrophobic fusion peptide. Presumably the presence of two highly hydrophobic domains within a single protein allows the fusion protein to bring the target and effector cells into close contact, facilitating membrane fusion (60).

The PI cells do not produce syncytia. This defect is not likely due to the elimination of the cytoplasmic tail of gp41, as previous reports have shown that major truncations of gp41's cytoplasmic tail do not abolish syncytium formation and actually enhance syncytium formation in some cell types (6, 11, 14, 25, 32, 33). Another reason for lack of syncytium-promoting activity of the PI cells is that they might express insufficient quantities of properly folded, processed, and/or oligomerized envelope glycoprotein at their surface, due either to the glycolipid anchor itself or to the nine carboxy-terminal residues added from the GPI anchor addition signal (24). The data, however, suggest that this may not be the case. The PI cells express more (Fig. 3) proteolytically processed (data not shown) glycoprotein at their surface than the WT cells do, and these GPI-anchored envelope glycoproteins bind CD4 (Fig. 2) and V3 loop antibodies (Fig. 3). The PI cells also produce envelope glycoprotein oligomers (Fig. 4). Although it remains to be determined exactly how the GPI-anchored Env oligomers compare with WT oligomers, it is generally believed that correct folding and oligomerization are required for cell surface expression (27).

A more interesting and plausible reason for the inability of

FIG. 5. Cocultures of EE (control) (A), WT (B), SEC (C), and PI (D) cells with HeLa-T4 cells. Only the WT cells produce syncytia with CD4⁺ cells.

the PI cells to induce syncytia is that GPI-anchored viral glycoproteins are inherently nonfusogenic. This possibility is supported by recent studies on a GPI-anchored influenza virus hemagglutinin which forms a trimer, binds target membranes, and changes conformation under fusion-inducing conditions like the wild-type molecule (29) does but does not execute a complete membrane fusion reaction (30).

Summary. The cell lines described in this report provide a stable and noninfectious source of membrane-anchored and secreted HIV-1 envelope glycoproteins that could be useful for a variety of studies of Env structure and function. Currently, the only available stable Env-expressing cell line (16), from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, expresses less Env at the cell surface than our wild-type Env-expressing cells do (36), and those cells do not form visible syncytia (36, 49). Our wild-type Env-expressing cells readily induce syncytia with CD4⁺ human cells, making them valuable reagents for fusion studies and fusion inhibitor assays. The GPI-anchored and secreted envelope glycoprotein-expressing cells will also be valuable for biochemical, biophysical, and immunologic studies of the Env ectodomain. Finally, a soluble oligomeric envelope glycoprotein could prove to be an important component of an HIV-1 vaccine, as an oligomeric Env complex would probably elicit a spectrum of antibodies different from that of monomeric gp120 or gp160. Not only have antibodies specific for oligomeric gp41 been identified in HIV-1-infected individuals (47), but also previous work on the human hepatitis subunit vaccine showed that aggregated or oligomeric forms of the

hepatitis virus surface antigen were critical for effective protective immunization (41, 56).

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