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Expression and Characterization of Glycophospholipid-Anchored Human Immunodeficiency Virus Type 1 Envelope Glycoproteins

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Four chimeric human immunodeficiency virus type 1 (HIV-1) *env* genes were constructed which encoded the extracellular domain of either the wild-type or a cleavage-defective HIV-1 envelope glycoprotein (gp160) fused at one of two different positions in *env* to a C-terminal glycosyl-phosphatidylinositol (GPI) attachment signal from the mouse Thy-1.1 glycoprotein. All four of the constructs encoded glycoproteins that were efficiently expressed when Rev was supplied in *trans*, and the two cleavable forms were processed normally to gp120 and a chimeric "gp41." The chimeric glycoproteins, in contrast to the wild-type glycoprotein, could be cleaved from the surface of transfected cells by treatment with phosphatidylinositol-specific phospholipase C, indicating that they were anchored in the plasma membrane by a GPI moiety. These GPI-anchored glycoproteins were transported intracellularly at a rate only slightly lower than that of the full-length HIV-1 glycoprotein and were present on the cell surface in equivalent amounts. Nevertheless, all four glycoproteins were defective in mediating both cell-cell and virus-cell fusion as determined by syncytium formation in COS-1–HeLa-T4 cell mixtures and *trans* complementation of an *env*-defective HIV-1 genome.

The *env* gene product of human immunodeficiency virus type 1 (HIV-1) is synthesized as a glycosylated polypeptide precursor, gp160, which undergoes cleavage during transport to the plasma membrane to yield two noncovalently linked subunits, gp41, the transmembrane glycoprotein (TM), and gp120, the surface glycoprotein (SU). The envelope glycoprotein of HIV-1 is essential for attachment of the virus particle to the target cell, fusion of the viral envelope with the plasma membrane, and formation of syncytia in infected cultures. Each of these functions involves an interaction between the glycoprotein and the CD4 receptor (see references 14 and 23 for reviews).

The TM glycoprotein contains an unusually long putative cytoplasmic domain which appears to be required for productive infection in most cell lines but not for syncytium formation (6, 10, 32). Mutations have defined the region from residue 684 to 705 as being necessary to anchor the protein in the membrane (1, 6, 15). This putative membrane-spanning domain is unusual in that it contains a highly conserved, charged basic residue in the middle of the long uncharged hydrophobic domain (26). Site-directed mutagenesis of this charged residue (arginine 696) and the uncharged residues from position 684 to 705 has demonstrated that the membrane-spanning domain is apparently not involved in the fusion mechanism beyond serving as a membrane anchor (9, 12). However, mutagenesis of a flanking charged residue (lysine 683) or substitution of four foreign residues for the arginine at position 696 within the putative membrane-spanning domain has been shown to dramatically affect syncytium formation and to block virus entry (12). Therefore, the role of the membrane-spanning anchor domain of the HIV-1 glycoprotein in the fusion event remains an intriguing question.

Many cellular glycoproteins are anchored in the plasma membrane through a glycosyl-phosphatidylinositol (GPI) moiety, rather than a membrane-spanning protein anchor. Thy-1.1, a glycoprotein of murine thymocytes and neurons, is a well-studied example of such a protein. The C-terminal 53 amino acids of the Thy-1.1 precursor protein have been shown to be sufficient to mediate the attachment of a GPI anchor to a chimeric protein (3), a process that involves the specific removal of a 31-amino-acid C-terminal peptide and the formation of an amide linkage between a C-terminal cysteine and an ethanolamine residue (22, 31). The GPI linkage can be cleaved at the cell surface by using phosphatidylinositol-specific phospholipase C (PI-PLC), which releases the protein in a soluble form into the medium (see references 8 and 21 for reviews).

In order to further define the polypeptide domains and membrane anchorage requirements involved in the oligomerization, transport, processing, and fusion properties of the HIV-1 glycoprotein, as well as the incorporation of glycoprotein into virions, we have expressed four chimeric HIV-1–Thy-1.1 glycoproteins which are GPI anchored. Such molecules might also facilitate the isolation of a soluble, oligomeric form of the HIV-1 glycoprotein suitable for structural analyses. In order to define the sequences proximal to the membrane-spanning domain that are important in the above processes, we replaced by gene fusion either 191 (HT-1) or 173 (HT-2) C-terminal amino acids of the wild-type HIV-1 glycoprotein with the 53-amino-acid Thy-1.1 GPI attachment signal. In addition, we made analogous constructs (HTC-1 and HTC-2, respectively) by using an *env* gene that encoded three amino acid substitutions (K-502→E [K502E], R504S, and K510E) near the C terminus of gp120 which prevent the processing of gp160 to gp120 and gp41 (7). The products of each of these constructs would be expected to retain 22 amino acid residues from the Thy-1.1 signal

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following GPI attachment. The added sequence includes an additional site for N glycosylation.

These chimeric glycoproteins were expressed in COS-1 cells at levels comparable to wild-type levels when a complete *rev* gene was supplied in *trans* by cotransfection and could be efficiently cleaved from the cell surface by treatment with PI-PLC. The rate of intracellular transport of these GPI-anchored HIV-1 glycoproteins was only slightly less than that of the full-length glycoprotein. Although the GPI-anchored glycoproteins could be detected on the cell surface in amounts comparable to wild-type glycoprotein, all were completely defective in mediating fusion as determined by assays of both syncytium formation and virus entry.

MATERIALS AND METHODS

Cell culture. COS-1 cells were obtained from the American Type Culture Collection. HeLa-T4 and H9 cells were obtained through the AIDS Reference and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and were originally contributed by Richard Axel and Robert Gallo, respectively. COS-1 and HeLa-T4 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. H9 cells were maintained in RPMI 1640 medium containing 15% fetal bovine serum.

Construction of plasmids. In order to create the chimeric HIV-1 *env*–Thy-1.1 genes, an *Ava*I site was introduced into the 3′ end of the *env* gene of the HXB2 strain of HIV-1 (27) at nucleotide 7796 (pHT-1 and pHTC-1) or 7850 (pHT-2 and pHTC-2) (Fig. 1A) by oligonucleotide-directed mutagenesis using the Altered Sites system from Promega as described previously (5). pThy-1.1, a cDNA clone encoding Thy-1.1 (16), was a gift from Ihor Lemischka (Princeton University). An *Xho*I linker (Boehringer Mannheim) was inserted into the 3′ flanking polylinker region downstream of the Thy-1.1 cDNA of pThy-1.1 following cleavage with *Bgl*II and blunt ending with the Klenow fragment of *Escherichia coli* DNA polymerase I (Fig. 1B). The resulting *Ava*I-*Xho*I fragment from pThy-1.1 encoding the 53 C-terminal amino acids of Thy-1.1 was substituted into both wild-type and cleavage-defective HXB2 *env* genes between the engineered *Ava*I sites and a unique *Xho*I site. Chimeric *Kpn*I-*Xho*I *env* gene fragments were then substituted into pSRHS, an HIV-1 *env* expression plasmid utilizing the late promoter and origin of replication from simian virus 40 and the poly(A) addition signal from the Mason-Pfizer monkey virus (6), for expression in COS-1 cells. The resulting constructs (pHT-1 and pHT-2, and pHTC-1 and pHTC-2, respectively) encode the ectodomain of gp160 fused at the C terminus to the GPI attachment signal from Thy-1.1 (Fig. 1C).

Glycoprotein expression and radioimmunoprecipitation. Each of the chimeric constructs was cotransfected with pRev1 (18, a gift from Marie-Louise Hammarskjold) into COS-1 cells by a modified calcium phosphate-mediated method (2). At 48 h posttransfection, the cells were starved in leucine-free DMEM for 1 h and pulsed for 30 min in leucine-free DMEM supplemented with [^{3}H]leucine (500 μCi/ml, 145 Ci/mmol; DuPont-NEN). The labeled cells were then either lysed or chased for 3 h in complete DMEM. The chase medium was collected, and the cells were lysed by a 10-min incubation on ice in lysis buffer (1% Triton X-100, 25 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1% sodium deoxycholate). Cellular debris was removed by centrifugation in a microcentrifuge for 5 min at 4°C. Lysates and chase media were immunoprecipitated by addition of HIV-1-positive human serum and incubation at 4°C for 1 h. The immune complexes were incubated for 30 min at room temperature with fixed *Staphylococcus aureus* and pelleted in a microcentrifuge. The pellets were washed three times in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS) and once in 20 mM Tris-HCl (pH 6.8) prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

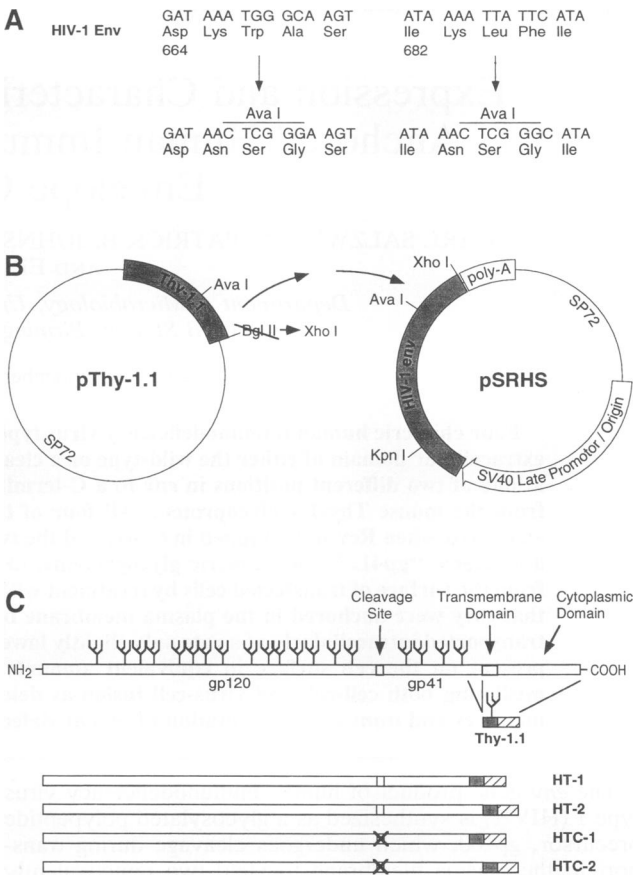

FIG. 1. Construction of chimeric HIV-1–Thy-1.1 glycoprotein mutants. (A) The nucleotide sequences encoding the indicated amino acids of the wild-type HIV-1 Env glycoprotein are shown at top; the mutations introduced to form the *Ava*I sites used for introducing the Thy-1.1 GPI attachment signal sequence and the resulting amino acid changes are indicated below. (B) Basic cloning strategy for pHT-1. The *Bgl*II site in pThy-1.1 was converted to an *Xho*I site. The resulting *Ava*I-*Xho*I fragment from pThy-1.1 was ligated into HIV-1 *env*, replacing the fragment between the new *Ava*I site created by site-directed mutagenesis and an *Xho*I site, using an intermediate vector (not shown). The *Kpn*I-*Xho*I fragment from the chimeric *env* was then substituted into pSRHS. (C) The wild-type glycoprotein is diagrammed at top with representative glycosylation sites. The transmembrane and cytoplasmic domains of gp41 were replaced with a 53-amino-acid, C-terminal GPI attachment signal from Thy-1.1 by gene fusion at each of the two new *Ava*I sites created in *env*. The resulting two constructs, HT-1 and HT-2, and their cleavage-defective sister constructs, HTC-1 and HTC-2, are diagrammed below. The hatched region represents the 31-amino-acid peptide which is cleaved concomitant with GPI attachment. The shaded region represents the 22 amino acids of Thy-1.1 which remain fused to the HIV-1 glycoprotein.

PI-PLC treatment. PI-PLC treatment was performed essentially as described previously by Crise et al. (3). Following labeling as described above, cells that had been chased

for 3 h were incubated for 15 min at room temperature in Ca^{2+}-Mg^{2+}-deficient phosphate-buffered saline (PBS) containing 25 mM EDTA to remove them from the dish. Cells were then washed and resuspended in DMEM containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.3). PI-PLC was added to the samples at a concentration of 17 U/ml (1 U = 1 μmol/min); this was followed by a 1-h incubation at 37°C. PI-PLC was generously provided by Martin Low (Columbia University). Both cells and media were collected following centrifugation and prepared as described above for immunoprecipitation and analysis by SDS-PAGE.

Endo H treatment. COS-1 cells were transfected and labeled as described above, except that they were starved in cysteine- and methionine-free DMEM for 30 min and labeled in cysteine- and methionine-free DMEM supplemented with [^{35}S]cysteine-[^{35}S]methionine (500 μCi/ml, 1,200 Ci/mmol; Expre^{35}S^{35}S; DuPont-NEN). Cells were lysed immediately following the pulse or were chased for 1, 2, or 4 h. Lysates were immunoprecipitated as described above, and the resulting washed pellets were boiled for 5 min in 0.04% SDS–200 mM 2-mercaptoethanol. The *S. aureus* was removed by pelleting at room temperature in a microcentrifuge, and the supernatant was divided into two tubes containing equal volumes of either 100 mM sodium citrate (pH 5.3)–2 mM EDTA–1 mM phenylmethylsulfonyl fluoride–200 μg of soybean trypsin inhibitor per ml–2 μg of leupeptin per ml–2 μg of pepstatin A per ml (mock treated) or the same buffer containing endoglycosidase H (endo H) (Boehringer-Mannheim) at a concentration of 50 U/ml (endo H treated). The samples were incubated for 16 h at 37°C, adjusted to 10 mM Tris-HCl (pH 6.8)–2% SDS–10% glycerol–0.1% bromophenol blue, and reboiled for 2 min prior to being loaded onto SDS-PAGE gels.

Syncytium assay and total cell immunofluorescence. COS-1 cells were trypsinized following transfection as described above, mixed with untransfected HeLa-T4 cells at a ratio of 1:10, and replated. At 48 to 72 h posttransfection, the cells were stained by the May-Grunwald-Giemsa technique as described previously (5), and the number and size of syncytia were quantitated.

For immunofluorescence staining, parallel cultures were seeded onto glass coverslips following mixing and were fixed at 48 to 72 h posttransfection with 95% ethanol–5% acetic acid at −20°C. These cells were stained for gp120 and gp160 expression by using a 1:25 dilution of a monoclonal antibody to the V3 loop (monoclonal 9284 from DuPont-NEN), followed after washing by a 1:25 dilution of Texas red-conjugated goat anti-mouse antibody (Southern Biotechnology Associates) as described previously (5).

Biotinylation of glycoproteins on the cell surface. COS-1 cells transfected as described above were treated with sulfo-NHS-biotin (Pierce) essentially as described previously by Lisanti et al. (20). Briefly, cells were washed five times at 48 to 72 h posttransfection with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-C/M) and then incubated with 0.5 mg of sulfo-NHS-biotin per ml in PBS-C/M for 30 min on ice. The cells were then incubated with DMEM for 10 min on ice to quench the reaction and were washed four times with PBS-C/M containing 20 mM glycine prior to lysis, immunoprecipitation, and SDS-PAGE as described above. The proteins were blotted onto nitrocellulose, and the membrane was blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at room temperature. The blot was washed with PBS-T and probed with a 1:2,500 dilution of horseradish peroxidase-conjugated streptavidin (Fisher Scientific) in PBS-T for 45 min at room temperature. The blot was washed again in PBS-T prior to enhanced chemiluminescence detection as described by the manufacturer (Amersham).

pHXBΔenvCAT complementation assay and incorporation of glycoproteins into virions. The pHXBΔenvCAT complementation assays were performed essentially as described previously by Helseth et al. (11). Briefly, COS-1 cells were cotransfected with pHXBΔenvCAT, pRev1, and each of the chimeric *env* constructs by using DEAE-dextran (1 mg/ml). At 60 h posttransfection, the supernatant was collected and passed through a 0.45-μm filter. A portion of the filtered supernatant was assayed for reverse transcriptase activity as described previously (5). The remaining filtered supernatant was used to infect H9 cells with equivalent amounts of virus as determined from the reverse transcriptase assays. At 60 h postinfection, cells were lysed, and a portion of the lysate was assayed for chloramphenicol acetyltransferase (CAT) activity by incubation with [^{14}C]chloramphenicol and acetyl coenzyme A, followed by thin-layer chromatography. The percent acetylation was determined by quantitating the radioactivity in each of the resulting spots with an AMBIS radioanalytic imaging system (AMBIS Systems). pHXBΔ envCAT was a gift from Joseph Sodroski.

For the detection of glycoproteins in virions, COS-1 cells were either cotransfected with pFNΔenvCAT, a construct identical to pHXBΔenvCAT except for an ~300-bp deletion in the *pol* gene (4), and pSRHS (wild type) or cotransfected with pFNΔenvCAT, pRev1, and the HTC-1 *env* construct by using calcium phosphate. At 60 h posttransfection, the cells were labeled for 2 h with [^{35}S]cysteine-[^{35}S]methionine (1 mCi/ml) as described above. Following a 10-h chase, the supernatant was filtered through a 0.45-μm filter. The filtered supernatant was then layered onto a 1-ml sucrose cushion (15% sucrose and 10 mM HEPES prepared in PBS) and centrifuged at 25,000 rpm for 2 h at 18°C in an SW41 rotor (Beckman Instruments). The resulting virus pellet was resuspended in lysis buffer and prepared as described above for immunoprecipitation and SDS-PAGE.

RESULTS

Expression of HIV-1–Thy-1.1 chimeric glycoproteins. The chimeric *env* constructs described above have the second coding exon of the *rev* overlapping reading frame deleted. Since the *rev* gene product is required for efficient expression of the glycoprotein (30), it was necessary to cotransfect cells with both the chimeric pSRHS-based constructs and pRev1, a complete *rev* cDNA clone under the transcriptional control of the cytomegalovirus immediate early promoter. Cotransfection mixtures were titrated against pRev1 to ensure that glycoprotein expression was optimal (data not shown). While the chimeric HIV-1 *env*–Thy-1.1 genes are still not expressed as highly as wild-type *env* in the pSRHS expression system, their products appear to be processed and transported normally in a pulse-chase analysis (Fig. 2A). Following a pulse-label, a band migrating slightly faster than the wild-type gp160 was seen for each of the chimeric proteins (gp160*). During the chase, HT-1 and HT-2 were cleaved to gp120 and a gp41–Thy-1.1 chimeric molecule (gp41*). The gp41* proteins from HT-1 and HT-2 showed the approximately 2-kDa size difference expected from the different fusion points (18 amino acids apart) within the HIV-1 *env* gene sequence. However, both migrated more slowly than might have been expected from the substitution of the shorter Thy-1.1 sequences (22 amino acids and an additional

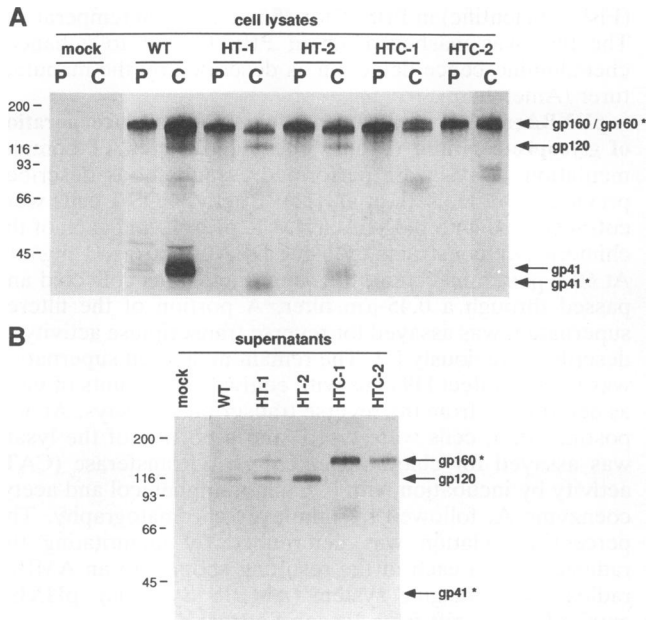

FIG. 2. Pulse-chase analysis of HIV-1–Thy-1.1 chimeric glycoprotein expression in COS-1 cells. COS-1 cells were transfected with simian virus 40-based expression plasmids encoding the wild-type HIV-1 glycoprotein (WT) and each of the chimeric products (HT-1, HT-2, HTC-1, and HTC-2). The chimeric constructs were cotransfected with pRev1, a cytomegalovirus-based Rev cDNA expression plasmid. The cells were metabolically pulse-labeled for 30 min with [^{3}H]leucine and chased for 3 h. Cell lysates (A) from both the pulse (P) and the chase (C) and the culture medium supernatants from the chase (B) were collected and immunoprecipitated with HIV-positive human serum prior to SDS-PAGE (8%) and fluorography. gp160, gp120, and gp41 denote the positions of the Env precursor, SU, and TM cleavage products, respectively. gp160* and gp41* indicate the analogous HIV-1–Thy-1.1 chimeric products. Molecular weight marker positions (in thousands) are shown at left. Mock-transfected cells were included as a negative control.

oligosaccharide side chain, following addition of the GPI anchor) for the longer HIV coding sequences (191 and 173 amino acids, respectively). Nevertheless, as addressed in more detail in the Discussion, the observed molecular weights (MWs) of the gp41* proteins from HT-1 (38,000) and HT-2 (40,000) are consistent with those predicted for the constructs, whereas that of wild-type gp41 (which migrates with an MW of 43,000 in these experiments) is significantly less than would be predicted.

The products of the HTC-1 and HTC-2 constructs were not cleaved to gp120 and gp41* following the chase, although a nonspecific cleavage of the V3 loop was observed (see below). A second band, migrating slightly slower than gp160*, was also immunoprecipitated from these cells. This band, most clearly visible in Fig. 2A, lane HTC-1 C, presumably represents the terminally glycosylated, uncleaved form of the chimeric gp160 present on the surface of expressing cells (see below).

Immunoprecipitation of the culture medium harvested from the chase plates showed that gp120 was shed into the medium from the wild-type, pSRHS-transfected cells (Fig. 2B, lane WT), consistent with the absence of a covalent linkage between gp120 and gp41. Similarly, primarily gp120 was found in the supernatant fraction of HT-2 (Fig. 2B, lane HT-2). In the case of HT-1, however, gp160*, gp120, and a trace of gp41* were found to be released from the cells during the chase (Fig. 2B, lane HT-1). We suspected that some membrane-bound chimeric proteins might be released by contaminating phospholipase activity from the fetal bovine serum in the culture medium, since a GPI-specific phospholipase D has been purified from mammalian serum (see reference 21 for a review). However, the HT-1 protein appears to be less stably associated with the plasma membrane than the HT-2 protein. Similarly, a significantly higher level of the uncleaved product from the HTC-1-transfected cells was shed into the medium than was observed with HTC-2 (Fig. 2B, lanes HTC-1 and HTC-2). It is possible, therefore, that following truncation of the external domain of gp41, the GPI anchor either is less able to secure the protein in the membrane or is more accessible to contaminating phospholipase.

It should also be noted that two bands migrating at MWs of approximately 85,000 and 70,000 appear predominantly in lanes corresponding to the cleavage-defective mutants HTC-1 and HTC-2. The sizes of these products are consistent with a proteolytic cleavage occurring within the V3 loop domain of the chimeric glycoprotein. We have previously observed heightened sensitivity to cleavage in this domain with full-length cleavage-defective mutants of gp160 (29).

The chimeric glycoproteins are GPI anchored and are transported to the cell surface. In order to determine whether the HIV-1–Thy-1.1 chimeric glycoproteins were GPI anchored and were transported to the cell surface, we determined whether these proteins could be cleaved by PI-PLC from the surface of cells cotransfected with each of the chimeric constructs and pRev1. PI-PLC has been used previously to show that up to 50% of the wild-type GPI-anchored Thy-1 glycoprotein produced by a cell can be cleaved from the cell surface (22).

The glycoprotein-expressing cells were pulse-labeled with [^{3}H]leucine and then chased for 3 h, at which time they were removed from the dish and either treated with PI-PLC or mock digested for 1 h at 37°C. Following removal of the solubilized proteins and lysis of the cells, both the cell lysates and supernatants were immunoprecipitated (Fig. 3A and B, respectively). A significant decrease in the intensities of both the gp120 and the gp41* bands was observed following digestion of cells expressing either HT-1 or HT-2, (Fig. 3A; compare HT-1, − and +, and HT-2, − and +, lanes). This decrease in cell-associated protein was accompanied by the appearance of gp120 and gp41* in the supernatant (Fig. 3B, lanes HT-1 [+] and HT-2 [+]), while during the 1-h mock digestion little protein was spontaneously shed into the medium (Fig. 3B, lanes HT-1 [−] and HT-2 [−]). Similar results were obtained with the cleavage-defective constructs HTC-1 and HTC-2. In each instance the amount of cell-associated protein decreased following digestion, and significant amounts of the solubilized gp160 ectodomain were released (Fig. 3A and B, lanes HTC-1 and HTC-2). In contrast, treatment of wild-type gp160-expressing cells with PI-PLC did not result in any release of the labeled protein into the medium (Fig. 3B, lane WT [+]).

It is interesting that a band corresponding to the gp160 ectodomain (gp160*) was also cleaved from the surface of both the HT-1- and HT-2-expressing cells. A more detailed analysis of this protein, as well as that released from HTC-1- and HTC-2-expressing cells, revealed that it migrated more slowly than the bulk of the protein that remained cell associated after PI-PLC digestion (Fig. 3C; compare the cell-associated [C] and solubilized [S] lanes) and corre-

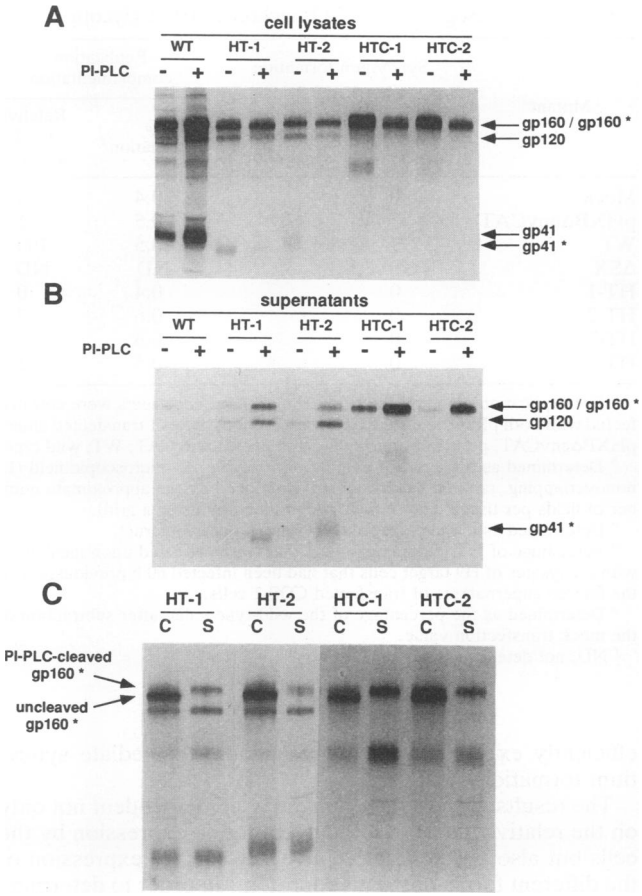

FIG. 3. Detection of GPI-anchored chimeric glycoproteins on the cell surface by cleavage with PI-PLC. COS-1 cells labeled with [^{3}H]leucine for 30 min were suspended in PBS following a 3-h chase, washed with serum-free medium, and incubated for 1 h at 37°C in serum-free medium (−) or serum-free medium containing PI-PLC (+). Both the cell lysates (A) and the culture medium supernatants (B) were collected and immunoprecipitated for SDS-PAGE (8%) and fluorography. WT, wild type. (C) Side-by-side comparison of products immunoprecipitated from the cell lysates (C) and the supernatants (S) following PI-PLC treatment, showing the differential mobilities of the cell-associated and solubilized products.

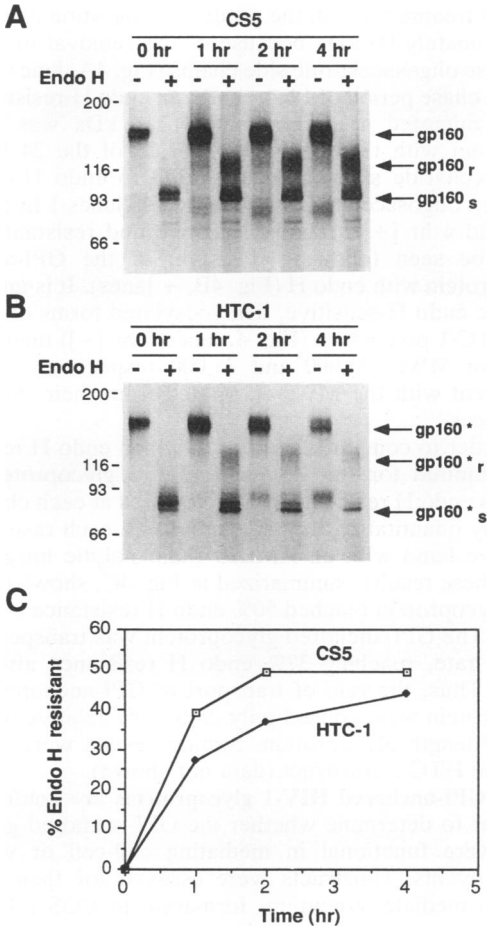

FIG. 4. Acquisition of endo H resistance by the GPI-anchored glycoprotein. Cells expressing either full-length cleavage-defective HIV-1 glycoprotein (CS5) (A) or HTC-1 (B) were lysed following a 30-min pulse (0 hr) with [^{35}S]cysteine-[^{35}S]methionine or following a 1-, 2-, or 4-h chase. The lysates were immunoprecipitated, boiled in buffer containing 0.02% SDS and 200 mM 2-mercaptoethanol, and incubated for 16 h at 37°C in the absence (−) or presence (+) of endo H. The samples were adjusted to 2% SDS and reboiled prior to SDS-PAGE (6%). gp160$_r$ and gp160*$_r$, endo H-resistant species; gp160$_s$ and gp160*$_s$, endo H-sensitive species. Molecular weight marker positions (in thousands) are shown at left. (C) The percent endo H resistance was determined by quantitating the radioactivity in each band with an AMBIS radioanalytic imaging system.

sponded to the upper band of the doublet described in the previous section. We interpret this result to mean that only the terminally glycosylated (higher-MW), surface-expressed gp160 is accessible to PI-PLC cleavage and that the bulk of this species is cleaved from the cell (compare, for example, lanes HTC-1, C and S in Fig. 3C). Moreover, this result indicates that a significant fraction of the HT-1 and HT-2 glycoprotein product is transported to the cell surface without being proteolytically cleaved to gp120 and gp41* in the Golgi.

The data presented in Fig. 3C also show that following cleavage, the cell-associated and solubilized gp41* proteins comigrate on SDS-PAGE. This indicates that removal of the lipid moiety from the GPI anchor does not significantly affect the apparent MW of gp41* in SDS-PAGE.

Rate of transport of the GPI-anchored HIV-1 glycoproteins. In order to determine whether the GPI anchor influenced the rate of intracellular transport of the chimeric glycoproteins relative to that of full-length gp160, we determined the rate at which they became resistant to endo H cleavage. This enzyme is unable to cleave from proteins oligosaccharide chains that have been trimmed by the mannosidases located in the *cis* and medial Golgi and thus provides a means to determine the rate of transport to this organelle (reviewed in reference 13). For this experiment, we compared the transport of natively and GPI-anchored cleavage-defective HIV-1 glycoproteins in order to simplify interpretation of the results. Cells expressing a full-length cleavage-defective (CS5) glycoprotein or the HTC-1 construct were pulse-labeled and then chased for 1, 2, or 4 h prior to lysis. Each of the cell lysates was then immunoprecipitated, divided into two tubes, and either treated with endo H or mock treated prior to SDS-PAGE.

As can be seen in Fig. 4, after a pulse-label, the full-length CS5 mutant HIV-1 glycoprotein was completely sensitive to

endo H treatment, with the product of digestion migrating at approximately 90 kDa because of the removal of its high-mannose oligosaccharide side chains (Fig. 4A, lane 0 hr [+]). After a chase period of 1 h or more, an endo H-resistant band which migrated at approximately 116 kDa was present, consistent with the modification of 13 of the 24 N-linked oligosaccharide side chains of gp120 to endo H-resistant, complex oligosaccharides (17) (Fig. 4A, lanes 1 hr [+], 2 hr [+], and 4 hr [+]). Similar sensitive and resistant species could be seen following digestion of the GPI-anchored glycoprotein with endo H (Fig. 4B, + lanes). It is interesting that the endo H-sensitive, deglycosylated forms of the CS5 and HTC-1 precursors (Fig. 4, lanes 0 hr [+]) migrate with apparent MWs (90,000 and 75,000 respectively) that are consistent with the MWs calculated from their amino acid sequences.

In order to compare the rates at which endo H resistance was acquired for the CS5 and HTC-1 glycoproteins, the percent endo H resistance was calculated at each chase time point by quantitating the radioactivity in each resistant and sensitive band with an AMBIS radioanalytic imaging system. These results, summarized in Fig. 4C, showed that the CS5 glycoprotein reached 50% endo H resistance after a 2-h chase. The GPI-anchored glycoprotein was transported at a similar rate, reaching 37% endo H resistance after a 2-h chase. Thus, the rate of transport of GPI-anchored HIV-1 glycoprotein was reduced only 25 to 30% relative to that of the full-length glycoprotein. Similar results were obtained with the HTC-2 construct (data not shown).

The GPI-anchored HIV-1 glycoproteins are nonfusogenic. In order to determine whether the GPI-anchored glycoproteins were functional in mediating cell-cell or virus-cell fusion events, constructs were assayed for their abilities both to mediate syncytium formation in COS-1–HeLa-T4 cell mixtures and to complement the entry–single-round replication of an *env*-deficient HIV-1 provirus in H9 cells. For the syncytium assay, COS-1 cells cotransfected with each of the HIV-1–Thy-1.1 chimeric constructs and pRev1 were mixed at a ratio of 1:10 with untransfected HeLa-T4 cells. The resulting cell mixtures were examined for the presence of syncytia 48 to 72 h later. No syncytia were seen with any of the GPI-anchored constructs, while numerous large syncytia were present in cells transfected with wild-type *env* (Table 1). Moreover, cells cotransfected with pRev1 and pSRHBΔSX, a construct expressing the *env* gene of the BH10 strain of HIV-1 that has the upstream coding region of *rev* deleted (4), produced a similar number and average size of syncytia (Table 1, ΔSX). Therefore, the lack of syncytia in the cultures expressing the GPI-anchored glycoproteins should not be due to decreased expression resulting from the cotransfection.

We further examined glycoprotein expression in this syncytium assay by staining parallel cultures for immunofluorescence with a monoclonal antibody specific for the V3 loop region of gp120 and gp160. The wild-type- and ΔSX-expressing cultures both contained numerous large, brightly fluorescing syncytia (Fig. 5, WT and ΔSX) with no brightly fluorescing single cells. This indicates that every COS cell expressing a significant level of wild-type glycoprotein, whether *rev* is provided in *cis* or in *trans*, is able to fuse adjacent cells. Conversely, the cultures expressing GPI-anchored glycoproteins contained numerous brightly fluorescing single and dividing cells (Fig. 5, HT-1, HT-2, HTC-1, and HTC-2), but again no syncytia were observed. Thus, even though the GPI-anchored glycoproteins were being efficiently expressed, they were unable to mediate syncytium formation.

TABLE 1. Fusogenicity of GPI-anchored HIV-1 glycoproteins

Mutant[a]	Syncytium formation		Replication complementation	
	No. of syncytia/plate[b]	No. of nuclei/syncytium[c]	% Acetylation[d]	Relative CAT activity[e]
Mock	0		0.4	0
pHXBΔenvCAT	ND[f]	ND	0.5	1
WT	299	38	10.5	100
ΔSX	190	31	ND	ND
HT-1	0		0.4	0
HT-2	0		0.6	2
HTC-1	0		0.6	2
HTC-2	0		0.5	1

[a] For replication complementation, chimeric *env* constructs were cotransfected with both pRev1 and pHXBΔenvCAT. Mock, pRev1 transfected alone; pHXBΔenvCAT, pRev1 cotransfected with pHXBΔenvCAT; WT, wild type.
[b] Determined as the average number of syncytia per microscopic field (15 nonoverlapping, random fields counted) multiplied by the approximate number of fields per tissue culture plate (determined by using a grid).
[c] Determined as the average from 15 syncytia per construct.
[d] Percentage of [^{14}C]chloramphenicol that was acetylated upon incubation with the lysates of H9 target cells that had been infected 60 h previously with the filtered supernatants of transfected COS-1 cells.
[e] Determined as the percentage of the wild-type value after subtraction of the mock transfection value.
[f] ND, not determined.

The results of the syncytium assay are dependent not only on the relative levels of total glycoprotein expression by the cells but also on the relative levels of surface expression of the different forms of the glycoprotein. In order to determine the approximate amount of each mutant glycoprotein relative to wild-type glycoprotein present on the cell surface, we selectively biotinylated proteins on the surface of COS-1 cells expressing each of the glycoproteins used in the syncytium assay. Following biotinylation and cell lysis, the glycoproteins were immunoprecipitated, separated by SDS-PAGE, blotted onto nitrocellulose, and detected by probing with horseradish peroxidase-conjugated Streptavidin followed by enhanced chemiluminescence.

As can be seen in Fig. 6, all four GPI-anchored glycoproteins were present on the cell surface at levels equivalent to that of wild-type glycoprotein. In addition to gp120, we also detected a considerable amount of uncleaved wild-type glycoprotein precursor (gp160), as well as uncleaved GPI-anchored glycoprotein precursor (gp160*) on the surface of cells expressing cleavable forms of the glycoprotein (Fig. 6, lanes WT and ΔSX, and HT-1 and HT-2, respectively). This result is consistent with that of the PI-PLC treatment experiment (see above) and demonstrates further that a significant fraction of the wild-type glycoprotein, as well as the HT-1 and HT-2 glycoproteins, is transported to the cell surface without being proteolytically cleaved to gp120 and gp41. The surface-expressed GPI-anchored gp160* detected in this experiment migrates as a single band. This again is consistent with the PI-PLC treatment experiment, which showed that only the terminally glycosylated (higher-MW) form of the glycoprotein is present on the cell surface (see above). The results of the syncytium quantitation, immunofluorescence staining, and surface biotinylation assays together demonstrate that the GPI-anchored glycoproteins are completely blocked in their ability to mediate cell-cell fusion between these two cell types.

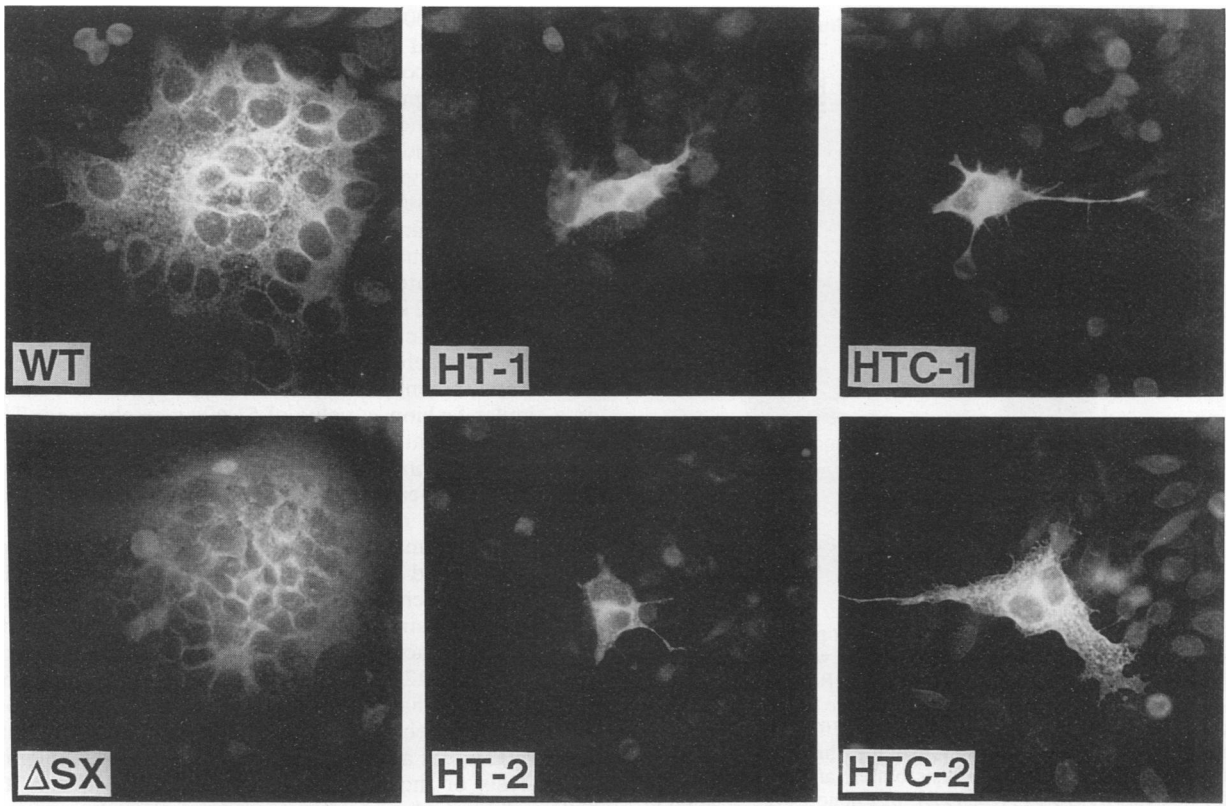

FIG. 5. Total-cell immunofluorescence of cells used in syncytium assay. COS-1 cells were transfected with pSRHS alone (WT) or cotransfected with pRev1 and pSRHBΔSX (ΔSX) or pRev1 and each of the chimeric constructs (HT-1, HT-2, HTC-1, and HTC-2). Immediately following transfection, the COS-1 cells were trypsinized and mixed at a ratio of approximately 1:10 with untransfected HeLa-T4 cells. At 48 h later, the cells were fixed with 95% ethanol–5% acetic acid and stained for immunofluorescence with a monoclonal antibody to the V3 loop and a Texas red-conjugated secondary antibody.

In order to determine whether the GPI-anchored glycoproteins could mediate entry of HIV-1 virions into T cells, we included the constructs in an *env* complementation assay described previously by Helseth et al. (11). This assay involves cotransfection of the *env* construct of interest with an HIV-1 proviral clone (pHXBΔenvCAT) containing a partially deleted *env* gene and a CAT reporter gene in place of the *nef* gene. The approximately 110-kDa product of this defective *env* gene is not incorporated into virions (data not shown). The virus produced by cotransfected COS-1 cells is used to infect $CD4^+$ target cells, which are then assayed for CAT activity 48 to 72 h later. Expression of CAT activity by the infected cells thus indicates that the glycoprotein encoded by the *env* construct is capable of mediating virus entry.

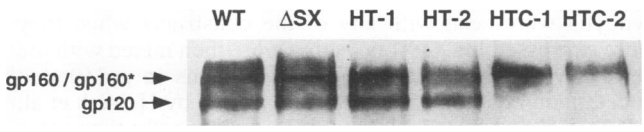

FIG. 6. Cell surface biotinylation. COS-1 cells transfected as for the syncytium assay were biotinylated at 48 h posttransfection by using sulfo-NHS-biotin. The cells were then lysed, and the glycoprotein was immunoprecipitated with HIV-positive human serum. The immunoprecipitates were run on an SDS-PAGE gel (8%) and blotted onto nitrocellulose. The biotinylated proteins were detected by probing the membrane with horseradish peroxidase-conjugated streptavidin, followed by enhanced chemiluminescence.

All four of our GPI-anchored mutants were incapable of complementing pHXBΔenvCAT in infecting H9 cells. Each yielded less than 2% of the CAT conversion obtained with a wild-type *env* control, and no significant difference between the cleavage-deficient (HTC-1 and -2) and the cleaved (HT-1 and -2) GPI-anchored constructs was observed (Table 1). These values were essentially identical to the levels of background conversion observed in the control transfection of pHXBΔenvCAT alone. Thus, the GPI-anchored HIV-1 glycoproteins are apparently defective in mediating virus-cell as well as cell-cell fusion.

The GPI-anchored HIV-1 glycoprotein is efficiently incorporated into virions. The results of the pHXBΔenvCAT complementation assay are dependent both on the ability of the mutant glycoprotein to mediate fusion of the viral and cell membranes and on the efficiency with which the mutant glycoproteins are incorporated into virus particles. We therefore analyzed labeled virus pelleted from the filtered COS-1 supernatants of a similar experiment for glycoprotein content. As shown in Fig. 7, gp120 can be seen associated with virus released from cells cotransfected with the wild-type *env* gene construct and pFNΔenvCAT (Fig. 7B, lane WT). The virions released from cells expressing the GPI-anchored HTC-1 product (gp160*) contained at least as much viral glycoprotein relative to the level of p24 *gag* core protein present (Fig. 7B, lane HTC-1). The cell lysates were

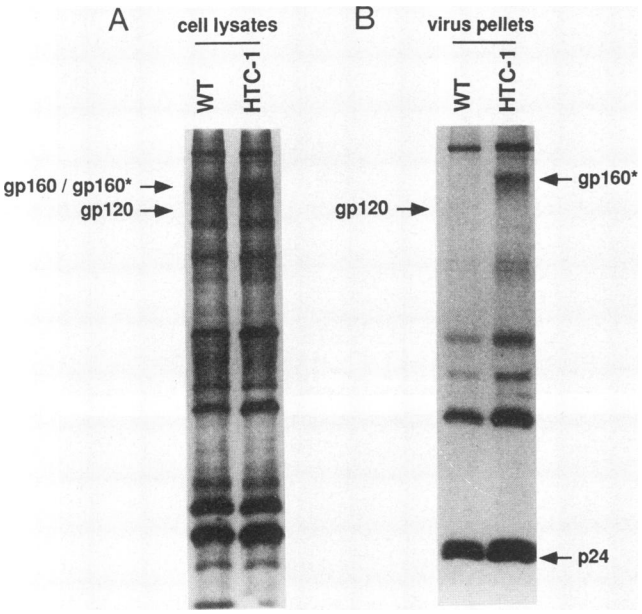

FIG. 7. Incorporation of GPI-anchored glycoproteins into HIV-1 virions. COS-1 cells were cotransfected either with pFNΔenvCAT and pSRHS (WT) or with pFNΔenvCAT, pRev1, and pHTC-1 (HTC-1). At 60 h posttransfection, the cells were labeled for 2 h with [^{35}S]cysteine-[^{35}S]methionine and chased for 10 h. The virus was pelleted from the filtered cell supernatants through a 1-ml 15% sucrose cushion, resuspended in lysis buffer, and immunoprecipitated for analysis by SDS-PAGE (9%). p24 denotes the position of the major capsid (CA) protein encoded by the *gag* gene.

also immunoprecipitated to ensure that equivalent amounts of glycoprotein were expressed in each culture (Fig. 7A). Thus, it appears that the GPI-anchored protein is incorporated into HIV-1 virions as efficiently as natively anchored glycoprotein.

DISCUSSION

In this study we have constructed four *env* constructs expressing chimeric HIV-1 glycoproteins in which the normal transmembrane and cytoplasmic domains were replaced by a GPI addition signal from Thy-1.1. These constructs express the glycoprotein efficiently when *rev* is supplied in *trans*. The resulting glycoproteins are anchored in the plasma membrane by a GPI anchor and can be solubilized from the cell surface by treatment with PI-PLC. This PI-PLC cleavage was highly efficient, since almost all of the terminally glycosylated glycoprotein species (presumably the only species present at the cell surface) was released into the supernatant by PI-PLC treatment.

A surprising result of these studies was the observation that the GPI-anchored gp41* proteins of HT-1 and HT-2 migrated on SDS-PAGE with a mobility similar to that of the wild-type gp41, despite the fact that sum totals of 169 and 151 amino acids (+22/−191 and +22/−173, respectively) had been deleted from the HT-1 and HT-2 gp41* proteins. Because the membrane-anchored and PI-PLC-solubilized gp41* proteins migrated with similar mobilities, this high apparent MW could not be attributed to the lipid anchor itself. Moreover, the additional glycosylation site present in the Thy-1.1 sequences, together with the oligosaccharide component of the anchor, would not be expected to increase the MW by 15,000. In the studies of Crise et al. (3), it was pointed out that the slightly larger apparent size of the GPI-linked, as compared with the wild-type, vesicular stomatitis virus G protein was consistent with the MW calculated following the replacement of 46 native amino acids with the 22-amino-acid-long, glycosylated Thy-1.1 sequence and GPI anchor. When the molecular masses of the HT-1 and HT-2 gp41* proteins were calculated from their amino acid sequences (176 and 194 amino acids, respectively) together with the additional mass contributed by five glycosylation sites (approximately 15 kDa) and the GPI anchor sequence (approximately 2 kDa), they were 37.1 and 39.5 kDa, respectively. These calculated values are very close to the 38- and 40-kDa values determined empirically from SDS-PAGE gels. In contrast, when the MW of wild-type gp41 was calculated in a similar fashion, a value of 51,600 was obtained. This is significantly greater than the empirically determined 43,000 MW for gp41 in these experiments. The basis for this discrepancy appears to be an effect on SDS-PAGE mobility imposed by the membrane-spanning domain and the long cytoplasmic domain of gp41, since a truncated "gp41" protein produced from Δ192-"gp160"-expressing cells has a mobility consistent with a molecular mass of approximately 31 kDa (4)—again, very close to the 29.2 kDa calculated from its amino acid sequence (17.2 kDa) and four oligosaccharide chains (12 kDa). Thus, it appears that the C-terminal region of gp41 that is deleted in the GPI-anchored proteins causes the protein to assume a more compact structure in SDS-PAGE that allows a more rapid, anomalous migration.

Analysis of the intracellular transport kinetics of these GPI-anchored glycoproteins indicated that their transport was only slightly reduced relative to that of the natively anchored glycoprotein. These findings are somewhat different from the results obtained when the same GPI attachment signal was fused to the vesicular stomatitis virus G glycoprotein. In this latter case, the effect on transport was much more significant, with the GPI-anchored protein being transported approximately eight times slower than the wild-type G protein (3). The altered transport kinetics of the GPI-anchored G protein are similar to those observed with G proteins lacking a cytoplasmic domain (28) and are consistent with this domain playing a role in efficient intracellular transport. In contrast, deletion of the entire cytoplasmic domain of the HIV-1 glycoprotein appeared to have no effect on intracellular transport of the protein in COS-1 cells (6, 10, 32), and the results we present here for the GPI-anchored "gp160" are consistent with both this domain and the membrane-spanning anchor domain being dispensable for efficient intracellular transport.

We have assessed the functionality of the chimeric GPI-anchored glycoproteins by assaying their abilities to mediate both cell-cell and virus-cell fusion. First, the GPI-anchored glycoproteins were assayed for their abilities to mediate syncytium formation in COS-1–HeLa-T4 cell mixtures. No syncytia were seen with any of the constructs when they were expressed in COS-1 cells that were then mixed with the permissive HeLa-T4 cells. Some mutations in gp160 that affect membrane fusion events were shown by Helseth et al. to affect syncytium formation more dramatically than virus entry (11). Therefore, we used the same pHXBΔenvCAT complementation system of Helseth et al. (11) to assay for virus entry mediated by the chimeric glycoproteins. The GPI-anchored glycoproteins proved to be completely defective in mediating virus-cell fusion in this assay, yielding acetylation values essentially identical to those obtained with the pHXBΔenvCAT vector alone. Thus, the GPI-

anchored glycoproteins appeared to be completely nonfusogenic. When we assayed the efficiency with which GPI-anchored glycoprotein was incorporated into virions, we found the HTC-1 glycoprotein to be incorporated into virions at least as efficiently as wild-type glycoprotein. Therefore, the 18- to 26-fold lower acetylation values for the GPI-anchored glycoproteins as compared with wild-type glycoprotein in the complementation assay could not be due to a low efficiency of incorporation into virions for these proteins.

The basis for the lack of HT-1 and HT-2 glycoprotein fusogenicity is not clear at present. While HT-1 lacks 18 amino acids from the putative extracellular domain of the HIV-1 glycoprotein which could be involved in the fusion event, this domain is intact in HT-2. In addition, preliminary experiments have demonstrated that both of these GPI-anchored glycoproteins are oligomeric (data not shown), suggesting that the conformation of the extracellular domain may not be significantly affected by the addition of the Thy-1.1 signal or the GPI anchor. Since previous studies have demonstrated that the cytoplasmic domain of gp41 is dispensable for intracellular transport of gp160 and for syncytium formation, it is possible that the membrane-spanning domain itself plays a critical role in membrane fusion. Indeed, Helseth et al. showed that a mutation of lysine 683 to an isoleucine residue inhibited both syncytium formation and complementation of pHXBΔenvCAT (12). Alternatively, the GPI anchor may not provide a sufficiently stable membrane association for the HIV-1 glycoprotein to function in bringing the two membranes sufficiently close together for membrane fusion to be initiated. It is clear from the pulse-chase experiments that the HT-1 and HTC-1 glycoproteins are shed into the medium of expressing cells at significant levels. While the HT-2 and HTC-2 proteins are more stable, they also appear to be shed from the membrane more readily than the wild-type anchored protein, which cannot be detected in the culture medium. However, this "shedding" could be due to glycoprotein release by a contaminating phospholipase in the culture medium containing fetal bovine serum.

It is also possible that the 22 amino acid residues and additional N-glycosylation site from Thy-1.1 which remain fused to the extracellular domain of the HIV-1 glycoprotein following addition of the GPI anchor, and/or the GPI anchor itself, may interfere with the fusion event. This interference could occur by blocking a conformational change that presumably must occur during fusion, by steric hindrance, or by positioning the glycoprotein complex too far outside the membrane bilayer, since there may be spatial constraints involved in bringing the two lipid bilayers together or since the fusion peptide may need to interact directly with the host membrane. Experiments are currently under way to address this issue by constructing analogous GPI-anchored HIV-1 glycoproteins which utilize a recently described minimal GPI attachment signal (19, 24, 25), thus eliminating any exogenous amino acid sequences.

While the GPI-anchored glycoproteins described above are nonfusogenic, we anticipate that these constructions will be useful in the production of quantities of a soluble form of HIV-1 glycoprotein suitable for structural analyses. Preliminary experiments have demonstrated that the GPI-anchored glycoprotein forms oligomers with sizes comparable to those of the full-length glycoprotein and that these oligomers can be detected in the culture supernatant after treatment with PI-PLC (data not shown). These constructs should be particularly useful in obtaining pure glycoprotein because they allow the molecule to be specifically cleaved from the cell surface by PI-PLC.

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