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Truncation of the Human Immunodeficiency Virus Type 1 Transmembrane Glycoprotein Cytoplasmic Domain Blocks Virus Infectivity

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Human immunodeficiency virus type 1 contains a transmembrane glycoprotein with an unusually long cytoplasmic domain. To determine the role of this domain in virus replication, a series of single nucleotide changes that result in the insertion of premature termination codons throughout the cytoplasmic domain has been constructed. These mutations delete from 6 to 192 amino acids from the carboxy terminus of gp41 and do not affect the amino acid sequence of the regulatory proteins encoded by *rev* and *tat*. The effects of these mutations on glycoprotein biosynthesis and function as well as on virus infectivity have been examined in the context of a glycoprotein expression vector and the viral genome. All of the mutant glycoproteins were synthesized, processed, and transported to the cell surface in a manner similar to that of the wild-type glycoprotein. With the exception of mutants that remove the membrane anchor domain, all of the mutant glycoproteins retained the ability to cause fusion of CD4-bearing cells. However, deletion of more than 19 amino acids from the C terminus of gp41 blocked the ability of mutant virions to infect cells. This defect in virus infectivity appeared to be due at least in part to a failure of the virus to efficiently incorporate the truncated glycoprotein. Similar data were obtained for mutations in two different *env* genes and two different target cell lines. These results indicate that the cytoplasmic domain of gp41 plays a critical role during virus assembly and entry in the life cycle of human immunodeficiency virus type 1.

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV) belong to a subgroup of retroviruses, the lentiviruses. This group of viruses differs from other retroviruses by producing a slowly progressing, long-term disease and by having a more complex genetic organization that includes several regulatory and accessory genes. One other important difference is the presence of an unusually long cytoplasmic domain in the viral glycoprotein. The envelope glycoprotein of HIV-1 is synthesized as a 160-kDa precursor that is cleaved intracellularly into the 120-kDa surface (SU) subunit and the 41-kDa membrane-spanning (TM) subunit (40). The SU subunit is responsible for the receptor binding function of the glycoprotein (28), and the TM glycoprotein anchors the SU/TM complex to the virion. At its N terminus, the TM glycoprotein contains an N-terminal fusion peptide. Toward the C terminus, it contains a membrane-spanning domain and a C-terminal domain that is exposed to the cytoplasm of infected cells and is referred to as the cytoplasmic domain (for a review, see reference 29). The cytoplasmic domain of lentiviruses differs from other retroviruses in that it consists of more than 150 amino acids; other retroviruses have cytoplasmic domains consisting of 20 to 50 amino acids.

While HIV-1, HIV-2, and SIV have a similar genetic organization, including Env proteins with cytoplasmic domains of similar sizes (16), they apparently have different requirements for this region in their replication. Many isolates of HIV-2 and SIV contain a stop codon that truncates the cytoplasmic domain to within 15 to 20 amino acids of the membrane-spanning domain. This has been shown to be a result of passage of the virus in tissue culture (17, 20). The development of a stop codon significantly increases the ability of the virus to replicate in vitro (20). Moreover, additional studies of an isolate which had a naturally occurring truncation showed that increasing the length of the cytoplasmic domain by removing the naturally occurring stop codon inhibited the replication of the virus in human cells (4). In contrast, despite the large number of HIV-1 isolates studied, to date only one infectious isolate of HIV-1 that contains a cytoplasmic domain reduced in size has been reported (35). Furthermore, the exact nature of the reduction in size of the TM protein of this isolate has yet to be clarified. So, while the data seem to support a growth advantage for the truncation of the cytoplasmic domain in HIV-2 and SIV isolates in vitro, there is a strong selection against any reduction in the length of the cytoplasmic domain in HIV-1.

Several studies aimed at defining the function of the large cytoplasmic domain of HIV-1 have been performed (12, 22, 24, 38). The earliest studies suggested that a deletion encompassing the last 14 nucleotides of the carboxy terminus of gp41 allowed the virus to replicate but abrogated the cytopathic effects of the virus (12). However, the deletion resulted in a frameshift and the addition of foreign amino acids to the carboxy terminus of gp41. Other studies have suggested that this effect may be cell-type specific (21, 24). Later studies used similar deletion mutants that resulted in frameshifts and the addition of foreign amino acids (24, 38). Moreover, some of these studies made use of extensive growth of infected cultures to propagate virus used in the studies. The recent information concerning the adaptation of SIV to extended in vitro culture (20) indicates that the results of studies which utilized extensive in vitro cultivation of HIV-1 mutants may need to be interpreted with caution.

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In other retroviruses, the requirement for the cytoplasmic domain varies. Rous sarcoma virus containing a glycoprotein lacking a cytoplasmic domain can efficiently infect cells (33), whereas deletion of the cytoplasmic domain of Mason-Pfizer monkey virus prevents glycoprotein incorporation into the virus even though it results in a glycoprotein that is more fusogenic (2). In other virus systems, for example, the vesicular stomatitis virus G protein, the cytoplasmic domain has been shown to be involved in intracellular transport of the glycoprotein (34), whereas truncation of the cytoplasmic domain of the hemagglutinin glycoprotein of influenza virus blocked infectivity without affecting incorporation of the glycoprotein into virions (36).

To address the role of the HIV-1 cytoplasmic domain in glycoprotein synthesis and maturation, as well as in virus replication and cytopathicity, we have inserted a series of premature termination codons into the region encoding the cytoplasmic domain of two isolates of HIV-1. These mutations resulted in the deletion of 6 to 192 amino acids from the carboxy terminus of gp41 and were constructed by changing a single nucleotide, therefore not disrupting the *tat* or *rev* open reading frames that overlap with the coding region for the cytoplasmic domain. Results from these studies show that in *env* genes from two different molecular clones, truncation of gp41 abrogated the ability of the mutants to infect T-cell lines. However, none of the mutations affected protein synthesis or transport, and all mutant glycoproteins that retained a membrane anchor were competent to cause fusion of CD4-expressing HeLa cells and T cells. These studies indicate a requirement for the carboxy terminus of gp41 to be intact for efficient HIV-1 replication in T cells.

MATERIALS AND METHODS

Cell culture and transfection. COS-1 cells were obtained from the American Type Culture Collection (Rockville, Md.). HeLa-T4, CEMx174, 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 (HeLa-T4), Robert Gallo (H9), and Peter Cresswell (CEMx-174). Cells were maintained in either Dulbecco's modified Eagle medium (DMEM) (GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum (COS-1 and HeLa-T4) or in RPMI 1640 (GIBCO) containing 15% fetal bovine serum (CEMx174 and H9). Transfections were performed with either a modified $CaPO_4$ technique (5) or DEAE-dextran at a final concentration of 1 mg/ml.

Oligonucleotide-directed mutagenesis and DNA manipulation. Mutations in the BH10 *env* gene were constructed by using the method of Zoller and Smith (42), and mutations in the HXB2 *env* gene were constructed by using the Altered Sites mutagenesis system (Promega, Madison, Wis.) essentially as we have described previously (8). DNA manipulation enzymes were obtained from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's directions or standard techniques (27). All mutations were confirmed by Sanger dideoxy sequencing, and in the case of the mutations in the BH10 *env* gene, the entire region subject to mutagenesis was sequenced to ensure that no second site mutations were created.

Plasmids and expression vectors. The pSRHS expression vector contains the HIV *env* gene under control of the simian virus 40 late promoter and the Mason-Pfizer monkey virus long terminal repeat to provide polyadenylation signals. The presence of the simian virus 40 origin of replication permits amplification of the plasmid on transfection into COS-1 cells. The details of this expression vector will be published elsewhere. The infectious molecular HIV-1 clone pHXB2D-gpt has been described elsewhere (12). To construct pFN, an expression vector containing a replication-defective HXB2 genome, an in-frame deletion of 109 amino acids was made in the *pol* gene by digestion with *Asp*718 to remove a 327-nucleotide fragment (nucleotides 3829 to 4157) and filling in the 5′ overhang with dGTP and the Klenow fragment of DNA polymerase. The remaining single-stranded regions were removed with S1 nuclease, and the plasmid was religated. The deleted genome was then subcloned by using *Hpa*I and *Xba*I in a plasmid containing the neomycin resistance cassette from pSV2neo (37). All plasmid DNAs were propagated in *Escherichia coli* DH-1 cells and purified by using cesium chloride gradients as described previously (27) with a TL100 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.).

Metabolic labeling of pSRHS-transfected cells. COS-1 cells were transfected with the pSRHS constructs by using DEAE-dextran. Two days after transfection, the cells were labeled with 125 μCi of [^{3}H]leucine in leucine-deficient DMEM for 30 min. At the end of 30 min, the [^{3}H]leucine was removed and replaced with leucine-containing medium, and the incubation was continued for an additional 3 h. The cells were lysed in lysis buffer (1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate in phosphate-buffered saline [PBS]) and incubated with serum from an HIV-1-infected individual, and 30 μl of a 10% suspension of fixed *Staphylococcus aureus* organisms was added. The immunoprecipitates were analyzed by SDS–8% polyacrylamide gel electrophoresis (PAGE) and then by fluorography.

Glycoprotein incorporation analysis. COS-1 cells in 100-mm-diameter plates were transfected with the pFN plasmid containing either the wild-type or mutant *env* gene. Two days after transfection, the cells were labeled with 750 μCi of [^{35}S]methionine-cysteine labeling mix (Protein Labeling Mix; Dupont NEN, Boston, Mass.) in methionine- and cysteine-deficient medium. The cells were labeled for 90 min, at which time the label was removed, complete DMEM containing 10% fetal bovine serum was added, and incubation was continued for 8 h. The supernatant was collected, and following removal of cellular debris by low-speed centrifugation, the supernatant was layered onto a 25% (wt/vol) sucrose cushion and centrifuged for 3 h in an SW41 rotor at 25,000 rpm. The pellet was resuspended in lysis buffer and immunoprecipitated as described above. Immunoprecipitates were analyzed by SDS–10% PAGE and then by fluorography.

HeLa-T4 fusion assay. HeLa-T4 cells were transfected with either the pFN or pSRHS plasmids containing the wild-type or mutant *env* genes by using the DEAE-dextran method. Two days after transfection, the cells were stained by the May-Grunwald/Giemsa technique. Briefly, the cells were washed with PBS, and 1 ml of May-Grunwald stain (0.25% in methanol) was added. The plates were incubated for 10 min, after which time an equal volume of distilled water was added to each plate. After an additional 10 min of incubation, the stain was removed and Giemsa stain (1/25 dilution of a 0.4% stock) was added for 30 min before the plates were washed with distilled water.

Infectivity assays. To assay for infectivity of mutant viruses, mutant *env* genes were cloned into the infectious clone pHXB2Dgpt by using the unique *Sal*I, *Bam*HI, or *Xho*I site. For the BH10 mutants, the entire BH10 *env* gene was first cloned into pHXB2Dgpt by using the unique *Sal*I

and *Xho*I sites to create pHXB.BH10. The appropriate mutant *env* fragment was then cloned into this construct. COS-1 cells were transfected with wild-type or mutant constructs and allowed to grow for 2 additional days. Supernatants were collected and assayed for reverse transcriptase (RT) activity as described below. The supernatants were adjusted with medium to equivalent RT values, and 1 ml of adjusted supernatant was used to infect 1 ml of medium containing 3×10^6 target cells. The cells were incubated for 2 h, and then 8 ml of complete medium was added. The cells were split 1:10 every 3 to 4 days as indicated in Fig. 2.

RT assay. RT assays were performed basically as previously described (8). Briefly, 25 μl of culture supernatant was incubated with 75 μl of a reaction cocktail {67 mM Tris-HCl [pH 8.0], 6.7 mM dithiothreitol, 6.7 mM $MgCl_2$, 200 mM KCl, 0.133% Triton X-100, 0.67 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid], 5 μCi of [^{35}S]-TTP (10 mCi/ml; Dupont NEN), 1.25 μg of poly(A) · (dT) (Boehringer Mannheim, Indianapolis, Ind.)}. The reaction mixture was incubated for 90 min at 37°C, at which time the reaction was stopped by the addition of 50 μl of 200 mM sodium PP_i and blotted onto NA45 paper (Schleicher & Schuell). The samples were quantitated by using a radioanalytical imaging system (AMBIS Systems Inc., San Diego, Calif.).

CAT analysis. A previously described method (15) uses the expression of a glycoprotein from a separate expression vector such as pSRHS to complement a genome that has a deletion in *env* and the chloramphenicol acetyltransferase (CAT) gene in place of the *nef* gene (15). The infectivity of the complemented virus can be measured by quantitating the amount of CAT activity present in cells infected with the virions produced from the cotransfection. This system can assay the effects of mutations in *env* independent of effects of the mutation on *rev* or *tat* and measures a single round of infection. For these complementation experiments, pHXBΔ envCAT and mutant *env* genes in pSRHS were cotransfected into COS-1 cells as described by Helseth et al. (15), and 48 h later, virus was harvested. For analyses of complementation, 10^7 H9 cells were infected as described above. Two days after infection, the cells were harvested and CAT activity was assayed by using [^{14}C]chloramphenicol as described elsewhere (1). The reaction was allowed to proceed for 45 min before the reaction was stopped. The products were subjected to thin-layer chromatography and exposed to film or quantitated with an AMBIS radioanalytical imaging system.

An additional control vector (pFNΔenvCAT) was constructed for these experiments. In pFNΔenvCAT, the *pol* gene in pHXBΔenvCAT was replaced with the *pol* gene of pFN described above. This resulted in a vector that, when cotransfected with a wild-type *env*-expressing vector, produces virus that can fuse with cells and undergo initial entry events but cannot proceed through reverse transcription.

RESULTS

Construction of mutants in BH10 and HXB2 *env* genes. In an effort to understand the functional role of the long cytoplasmic domain of HIV-1, we have inserted a series of point mutations into the 3′ (C-terminal domain) of the *env* gene from both the BH10 and HXB2 viral genomes. The region of the *env* gene that codes for the carboxy terminus of gp41 also contains reading frames for the second exon of *rev* and *tat*. Therefore, to prevent altering these other gene products, site-directed mutagenesis was utilized to create

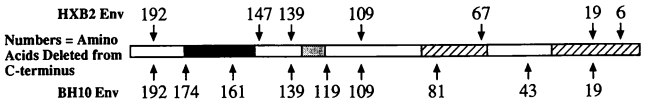


FIG. 1. Schematic diagram of mutation in the cytoplasmic domain of gp41 showing the locations of the termination codons inserted by using oligonucleotide-directed mutagenesis. The arrows indicate the locations of the mutations constructed in both the HXB2 *env* gene (above) and the BH10 *env* gene (below). The numbers refer to the number of amino acids deleted from the C terminus of gp41. See Table 1 for the exact amino acid changed. The putative membrane-spanning region (■), the hydrophilic region (▩) previously described (19), and the proposed amphipathic helices (▨) (39) are also denoted.

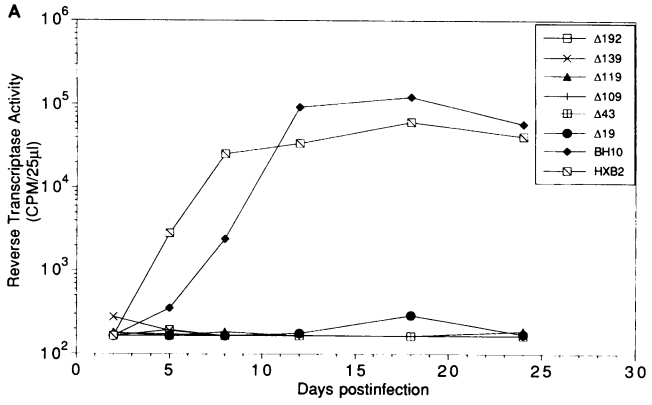
single nucleotide changes that would insert stop codons in *env* but that would not affect *rev* and *tat*. These point mutations introduce premature termination codons into the *env* open reading frame and result in the synthesis of progressively truncated proteins. Some of the mutations were designed to confirm the location of the membrane-spanning domain and define a role for the highly conserved hydrophilic domain (19) (Fig. 1; Table 1). The mutation that deletes 139 amino acids (Δ139) mimics the spontaneously arising termination codon found in SIV and HIV-2 isolates discussed above, and the Δ19 and Δ67 mutations were designed to disrupt the amphipathic helices that have been proposed for this region (39). All of the mutants were confirmed by DNA sequencing, and in the case of the BH10 mutations, the entire region mutagenized was sequenced to ensure that no second site mutations had been introduced. The mutant *env* genes were subsequently cloned into two expression vectors as well as into the infectious molecular clone pHXB2Dgpt (12). Since the molecular clone BH10 lacks the 5′ long terminal repeat, it is unable to produce infectious virions. Therefore, for infectivity studies involving mutations of the BH10 *env* gene, the entire *env* gene from BH10 was cloned into the HXB2 background and is designated HXB2.BH10. This construct also provides a functional *vpu* gene that is absent from the parental pHXB2Dgpt clone.

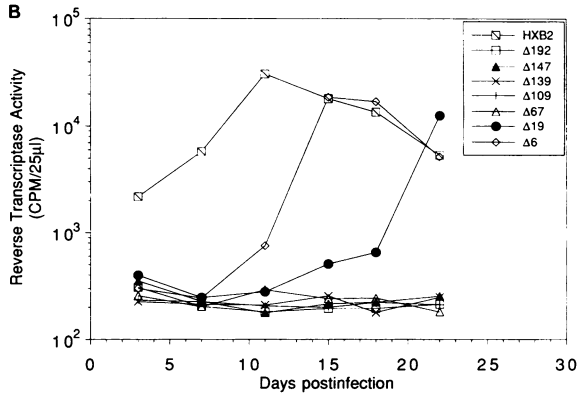
Infectivity of mutant HIV-1 genomes with truncations in the

TABLE 1. Summary of cytoplasmic domain mutants

Wild type or mutation in[a]:		Mutant designation[b]	Release of virions	Membrane-anchored gp160	Fusion	Infectivity of virions
BH10	HXB2					
K625→*	K625→*	Δ192	+	−	−	−
K683→*		Δ174	+	−	−	−
R696→*		Δ161	+	D[c]	−	−
	R709→*	Δ147	+	+	+	−
Q718→*	Q718→*	Δ139	+	+	+	−
G738→*		Δ119	+	+	+	−
L748→*	L748→*	Δ109	+	+	+	−
	W790→*	Δ67	+	+	+	−
L814→*		Δ43	+	+	+	−
R838→*	R838→*	Δ19	+	+	+	±
	L851→*	Δ6	+	+	+	±
Wild type			+	+	+	+
	Wild type		+	+	+	+

[a] The mutation is indicated by the amino acid location. *, conversion to stop codon.
[b] The mutant designation reflects the number of amino acids deleted.
[c] D, degraded.





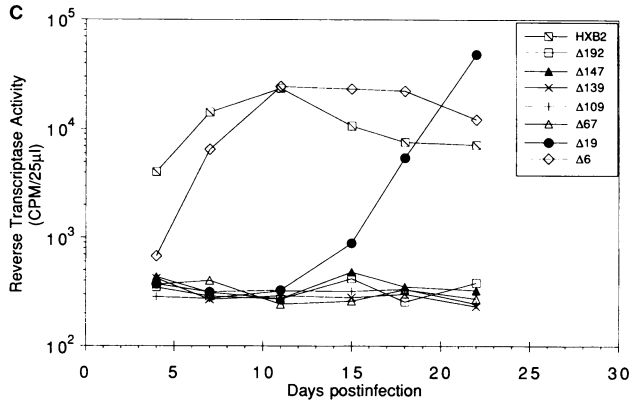


FIG. 2. Infectivity of virus containing truncations in the cytoplasmic domain of gp41. COS-1 cells were transfected with wild-type or mutant genomes. The supernatants were harvested and used to infect T cells as described in Materials and Methods. At the times indicated, aliquots of culture medium were removed and assayed for RT activity. (A) Virus from COS-1 cells transfected with HX B.BH10 wild-type and mutant genomes was used to infect H9 cells; virus from COS-1 cells transfected with HXB2gpt wild-type and mutant genomes was used to infect H9 (B) or CEMx174 (C) cells.

cytoplasmic domain of gp41. Initial studies were performed by using the mutations in the BH10 *env* gene. These mutations resulted in the truncation of 19 to 192 amino acids from the C terminus of gp41 (Fig. 1). The ability of these mutations to infect H9 cells was assayed by transfection of the molecular clone containing the mutant *env* gene into COS-1 cells. Two days after transfection, the virus-containing supernatant was harvested and assayed for RT activity. The supernatants were adjusted with medium to contain equivalent amounts of RT activity and used to infect H9 cells as described in Materials and Methods. To monitor the infection, aliquots of infected cultures were removed at the times indicated in Fig. 2A and assayed for RT activity. Both the parental pHXB2Dgpt construct and pHXB.BH10 produced virus that spread rapidly through the culture as detected by the increase in RT activity (Fig. 2A) of the culture supernatant. All of the other mutant viruses were unable to establish an infection when assayed in this manner. However, in other experiments, including coculture experiments, the Δ19 mutant virus showed a delayed infectivity, with RT activity becoming evident after 10 to 15 days (data not shown).

To confirm that the results were not influenced by the construction of the hybrid HXB2.BH10 genome, similar experiments were performed with the mutant HXB2 glycoprotein genes after reintroduction into the pHXB2Dgpt genome. Following transfection of the mutant genomes into COS-1 cells, the culture medium from each of the transfected cultures was adjusted with medium to contain equivalent amounts of RT activity. This was used to infect H9 and CEMx174 cells as described in Materials and Methods. At the times indicated in Fig. 2B, an aliquot of culture medium was removed for RT analysis and the infected cultures were split. As shown in Fig. 2B, when the wild-type virus was used to infect H9 cells, the virus spread rapidly through the culture, as indicated by the rapid increase in RT activity. The Δ192 mutant, which codes for a secreted glycoprotein and therefore represents a negative control, showed no increase in RT activity over the course of the infection. All of the other mutants, with the exception of Δ6 and Δ19, also showed no increase in RT activity over the course of the experiment. The RT activity of the culture infected with the Δ6 and Δ19 mutant viruses increased during the experiment, but the infection lagged behind that with the wild-type virus by approximately 10 days for Δ6 and by more than 20 days for Δ19. A similar pattern of infectivity is seen for CEMx174 cells (Fig. 2C), except in these cells, infection with Δ6 lagged behind that with the wild-type virus by only 2 to 3 days while Δ19 again took over 2 weeks to produce similar levels of RT activity. Similar results were obtained when the transfected COS-1 cells were cocultured with H9 cells (data not shown).

In these experiments, the cultures were infected with an amount of virus equivalent to 1,000 to 10,000 tissue culture infective doses of wild-type virus, as determined by serial 10-fold dilutions of the virus released from transfected COS-1 cells (data not shown). Therefore, truncating the cytoplasmic domain of gp41 by even as few as 19 amino acids can greatly affect or, with longer truncations, completely block the ability of the virus to infect both H9 and CEMx174 cells, even when they were infected with large amounts of virus.

During further studies of the synthesis and processing of the mutant glycoproteins, it became obvious that the BH10 glycoprotein was synthesized and processed at a significantly lower level than the HXB2 glycoprotein. Given these differences, mutations in the HXB2 *env* gene were selected for further study of the basis for the block in infectivity of these cytoplasmic domain truncation mutants.

Envelope protein synthesis and processing of HXB2 truncations. As shown in Fig. 1, a series of seven mutations that truncated 6 to 192 amino acids from the carboxy terminus of gp41 was constructed in the HXB2 *env* gene. To study the effects of these mutations on the synthesis and processing of

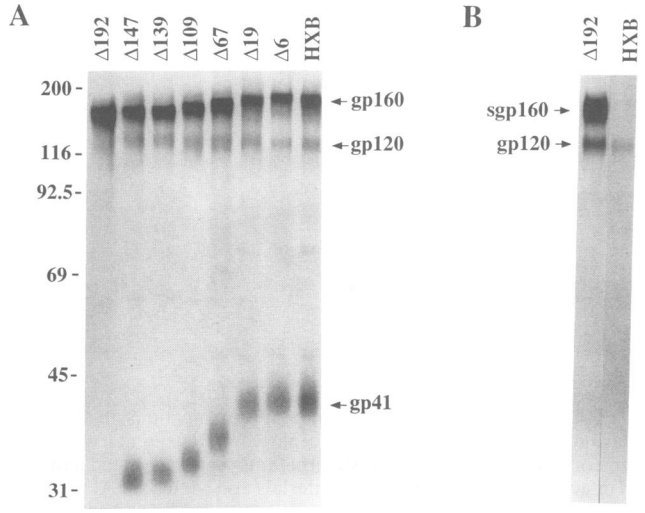


FIG. 3. Synthesis and processing of mutant glycoproteins. COS-1 cells were transfected with the pSRHS expression vector containing either wild-type or mutant *env* genes. The transfected cells were labeled with [^{3}H]leucine and immunoprecipitated with serum from an HIV-1-infected individual. Markers on the left show the molecular masses in kilodaltons, and the positions of the glycoprotein precursor and cleavage products are shown on the right in panel A.

the glycoproteins, the mutant *env* genes were inserted into the simian virus 40-based expression vector pSRHS. This vector utilizes the simian virus 40 late promoter to drive transcription of the *env* gene and includes the long terminal repeat of Mason-Pfizer monkey virus to provide polyadenylation signals. It has been shown to direct the synthesis of high levels of glycoprotein when transfected into COS-1 cells (8). Two days after transfection of the wild-type and mutant *env* expression plasmids, the cells were metabolically labeled for 30 min with [^{3}H]leucine and then further incubated for 3 h in the presence of unlabeled leucine. The cells were lysed, and the cell lysates were immunoprecipitated with serum from an HIV-1-infected individual. The use of [^{3}H]leucine allows the visualization of gp41 since the majority of leucine residues in gp160 are in the gp41 region. The lane labeled HXB in Fig. 3A shows the expression of the wild-type envelope glycoprotein in this system. The positions of the glycoprotein precursor, gp160, as well as the cleavage products gp120 and gp41 are indicated to the right of the figure. A relatively small amount of the HIV-1 glycoprotein precursor is cleaved to the mature products (40), and similar processing kinetics are seen with the pSRHS vector. Both the gp120 and gp41 subunits migrate as broad bands because of terminal glycosylation of the proteins. The increased mobility of the truncated gp41 molecules corresponds to the expected molecular weight reduction resulting from the insertion of the stop codons (Fig. 3A). None of the mutations appears to affect the synthesis, carbohydrate processing, or cleavage of the truncated glycoproteins (Fig. 3A). Since cleavage of the glycoprotein precursor to the gp120 and gp41 subunits occurs late in the Golgi apparatus, the mutations do not appear to alter the transport of the glycoprotein. On the basis of the results of others (10, 22), the Δ192 mutant would be expected to produce a glycoprotein that lacks a membrane-spanning domain and thus would be expected to be secreted into the culture medium. This is confirmed by the lack of cell-associated gp120 (Fig. 3A, lane labeled Δ192) and the release of a large amount of soluble glycoprotein into the culture medium. This was present in the form of both the uncleaved secreted gp160 (sgp160) precursor and the gp120 cleavage product (Fig. 3B, lane labeled Δ192). The gp120 from wild-type transfected cells is released at much lower levels into the culture medium, presumably because of a labile SU/TM association (Fig. 3B, lane labeled HXB). No gp160 is seen in the medium of wild-type transfected cells.

Fusion capacity of truncated glycoproteins. To test whether truncation of the cytoplasmic domain blocked the ability of the HIV-1 glycoproteins to fuse CD4-expressing cells, we utilized a HeLa-T4 syncytium-forming assay (11). In this assay, HeLa cells that constitutively express the CD4 molecule (26) were transfected with either the pSRHS expression vector or the pFN vector described above.

Initial studies were performed by utilizing the pSRHS vector, which expresses the glycoprotein in the absence of other HIV-1 structural proteins. When the wild-type glycoprotein is expressed in HeLa-T4 cells by using this vector, large syncytia consisting of 20 to 30 nuclei are produced (Fig. 4a, panel A). Interestingly, truncation of the cytoplasmic domain by 109 amino acids produced syncytia larger than those produced by the wild type. The Δ109 truncation increased the average number of nuclei per syncytium to 145% of the wild-type number (Fig. 4a, panel C; Table 2). However, no difference was seen for the Δ67 or Δ147 mutants (Fig. 4a, panels B and D) or for most of the other truncation mutants (Table 1). The only exception was the Δ19 mutation, which on the average resulted in syncytia that were smaller than those produced by the wild type (Table 2). This is likely due to a decreased amount of glycoprotein on the cell surface; a result of a more rapid turnover of the glycoprotein (see below).

The mutant glycoproteins were then expressed from the pFN vector and assayed for fusion in HeLa-T4 cells. This vector contains the entire HIV-1 genome, but a small deletion has been made in *pol* to render the virions noninfectious. As can be seen in Fig. 4b, results similar to those obtained with the pSRHS vector were also obtained with the pFN vector. As expected, the wild-type glycoprotein produced large syncytia (Fig. 4b, panel A), whereas the Δ192 mutant, which expresses a secreted glycoprotein, was unable to produce syncytia (Fig. 4b, panel H). The only mutant which gave different results with the pSRHS and pFN vector systems was the Δ109 mutant, which produced syncytia similar in size to those of the wild type when expressed from the pFN vector (Fig. 4b, panel E).

The HeLa-T4 assay provides a highly quantitative measure of syncytium-forming ability; nevertheless, we have observed qualitatively similar results when COS-1 cells transfected with either vector were overlaid with H9 cells and syncytium formation was scored after 24 h (data not shown).

Viral protein expression. Since all of the mutations were single nucleotide changes, we were able to avoid changing any amino acids in the overlapping *tat* or *rev* reading frames. Therefore, we were able to study the effects of the mutations and explore the basis for the lack of infectivity of these mutants in the context of other viral proteins. The mutant *env* genes, cloned into the infectious molecular clone pHXB2Dgpt, were transfected into COS-1 cells, and at 48 h posttransfection, the cells were labeled with [^{3}H]leucine for 30 min. Following a 4-h chase in complete unlabeled medium, the cells were lysed and immunoprecipitated as de-

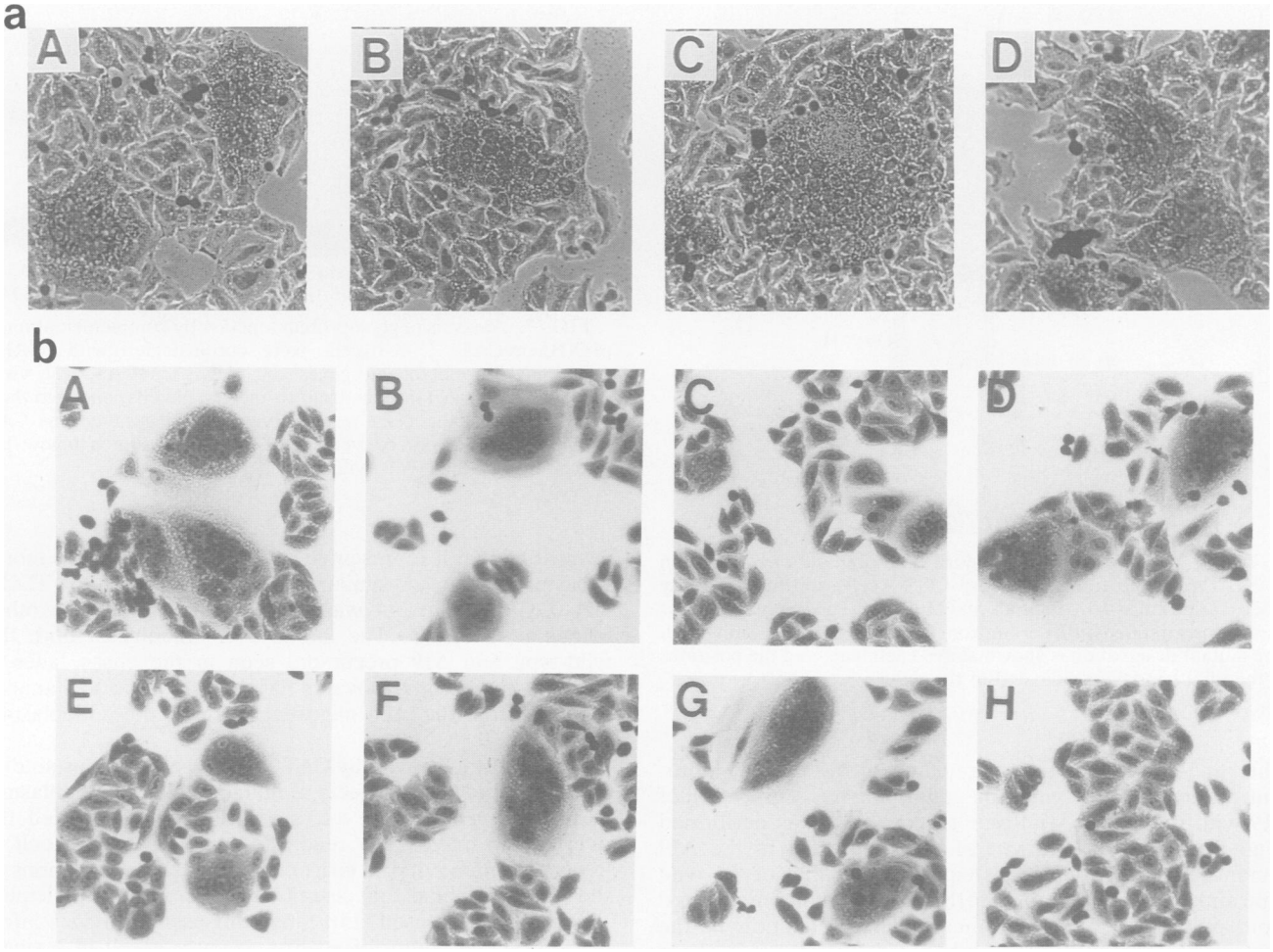


FIG. 4. Fusion assay of truncated glycoproteins expressed from the pSRHS or pFN expression vectors. HeLa-T4 cells were transfected with either the pSRHS or pFN expression vectors containing the mutant or wild-type *env* gene. Two days after transfection, the cells were stained as described in Materials and Methods. (a) Fusion assay of pSRHS-transfected cells. Panels: A, wild type; B, Δ67; C, Δ109; D, Δ147. (b) Fusion assay of pFN-transfected cells. Panels: A, wild type; B, Δ6; C, Δ19; D, Δ67; E, Δ109; F, Δ139; G, Δ147; H, Δ191.

scribed in Materials and Methods. The immune complexes were analyzed by SDS–10% PAGE and visualized by fluorography. The pattern of viral glycoproteins and Gag proteins immunoprecipitated from cells transfected with the wild-type pHXB2Dgpt is shown in Fig. 5 (lane labeled HXB). After the pulse-chase labeling, gp160, gp120, and gp41 and the viral *gag* gene products p55, p41, and p24 can be immunoprecipitated. A similar pattern of viral proteins was observed when the mutant pHXB2 constructs were analyzed. As with the pSRHS vector, the mutations resulted in the immunoprecipitation of progressively smaller gp160 and gp41 moieties. The processing of the glycoprotein was similar to that of the wild type in all of the mutants except Δ19 and Δ139. For Δ139, a decrease in the amount of cell-associated gp120 was seen (Fig. 5, lane labeled Δ139). This decrease in gp120 appeared to be the result of an inhibition of processing of gp160 since there was no detectable increase in the amount of gp120 shed into the culture medium (data not shown). This defect in processing of the Δ139 mutant was not seen when the mutant *env* gene was expressed in the pSRHS expression vector. However, the same inhibition of processing was seen when the analogous mutation in the BH10 Env protein was also analyzed in the context of the viral genome (data not shown), making it unlikely that the phenotype was the result of a second site mutation.

In the context of the viral genome, the Δ19 mutation results in significantly lower levels of cell-associated Δgp160, gp120, and Δgp41 (Fig. 5, lane labeled Δ19). To analyze

TABLE 2. Syncytium formation on transfection into HeLa-T4 cells

Mutant	pFN		pSRHS	
	Avg[a]	% of WT[b]	Avg[a]	% of WT
WT	18.3	100	27.8	100
Δ6	17.4	95.1	26.3	94.6
Δ19	8.4	45.9	8.8	31.7
Δ67	17.9	97.8	23.6	84.9
Δ109	17.2	94.0	40.4	145.0
Δ139	20.8	113.6	23.9	86.0
Δ147	18.0	98.4	28.0	100.7

[a] Average number of nuclei per syncytium. The nuclei of twenty syncytia were counted for each mutant.
[b] WT, wild type.

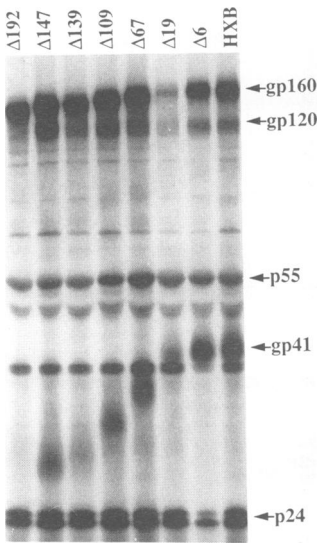


FIG. 5. Viral protein expression. The mutant *env* genes in pHXB2Dgpt were transfected into COS-1 cells and metabolically labeled as described in Materials and Methods. The cell lysates were immunoprecipitated with serum from an HIV-1-infected individual. The mutant designation is shown above each lane, and the positions of the viral bands are indicated to the right.

whether this represented degradation of the Δ19 glycoprotein, a time-course experiment comparing the degradation of Δ19 with that of the wild-type glycoprotein was performed (Fig. 6). While the amount of $p55^{gag}$ protein in the Δ19 mutant-transfected cells was equivalent to that of the wild type and similar amounts of gp160 were immunoprecipitated from pulse-labeled cells, a nearly 90% reduction in the amount of glycoprotein was observed over the 4-h chase period (Fig. 6). Moreover, this rapid turnover of the precursor was not accompanied by higher levels of gp120 and gp41,

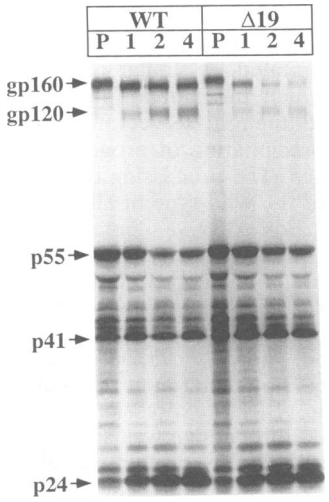


FIG. 6. Degradation of the Δ19 mutant glycoprotein in a time-course experiment of COS-1 cells transfected with pFN (containing the wild-type *env* gene [WT]) or pFNΔ19 (Δ19). The cells were pulse-labeled (lane P) or chased for the number of hours indicated above each lane. The positions of the viral proteins are shown to the left.

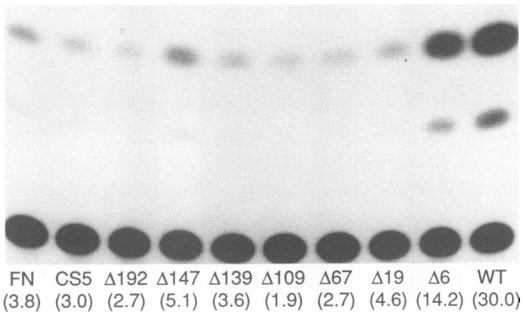


FIG. 7. Analysis of glycoprotein function by complementation of pHXBΔenvCAT. COS-1 cells were cotransfected with pSRHS containing the mutant *env* genes and pHXBΔenvCAT. The virus containing supernatant was used to infect H9 cells, and two days after infection, the cells were harvested and assayed for CAT activity. The percent conversion (% conv.) is shown below the mutant designation. WT, wild type.

suggesting that it represented degradation rather than more-rapid processing. A similar effect was seen for the HXB.BH10.Δ19 mutant but was not seen for any of the other truncation mutants. The increase in mobility of both the wild-type and Δ19 precursors seen in the chase lanes is consistent with the removal of terminal glucose and mannose residues from the core oligosaccharide in the endoplasmic reticulum.

Analysis of infectivity by CAT expression. Previous studies have reported on the effects of mutations in the cytoplasmic domain of gp41. However, since they often affected the overlapping *tat* and *rev* reading frames, it was difficult to assay the infectivity of virus containing these mutations in the same manner as that described above, and a complementation assay was used (15). Since this assay provides information on the ability of a virus to enter a cell in a single replication cycle, the cytoplasmic domain truncations were assayed for their ability to complement the pHXBΔenvCAT vector. To assay the cytoplasmic domain mutants, the pHXBΔenvCAT and pSRHS constructs were cotransfected into COS-1 cells. Two days after transfection, the culture supernatants were assayed for RT activity, and equivalent amounts of RT were used to infect H9 cells. After an additional 2-day incubation, the H9 cells were assayed for CAT activity. When the assay was performed with the pFNΔenvCAT control, a significant amount of background conversion could be detected (Fig. 7, lane labeled FN). Similarly when a nonfusogenic, cleavage-defective glycoprotein described elsewhere (9) was used to complement pHXBΔenvCAT, a small amount of CAT activity was observed in the H9 cells (Fig. 7, lane labeled CS5). These two controls were necessary to determine the background level of CAT activity. Complementation of pHXBΔenvCAT with the wild-type glycoprotein resulted in the expression of CAT in H9 cells that yielded 30.0% conversion of the substrate. Consistent with the infectivity results presented above, only the Δ6 mutant glycoprotein yielded CAT activity significantly above that of the negative controls (14.2%; Fig. 7). All of the other mutations showed a decreased level of CAT activity that approached or was less than the background produced by the controls.

Incorporation of truncated glycoproteins into virions. To determine if the truncated glycoproteins were incorporated into virions, we utilized the pFN variant of pHXB2Dgpt. This construct, with a small deletion in the *pol* gene,

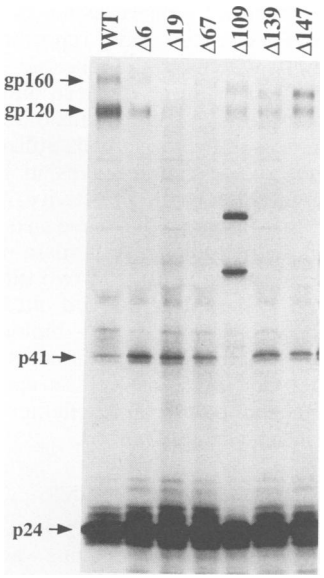


FIG. 8. Incorporation of mutant glycoprotein into virions. Virus-containing supernatants from metabolically labeled COS-1 cells transfected with wild-type (WT) or mutant pFN constructs were centrifuged through a 25% sucrose cushion. The virus pellets were recovered and immunoprecipitated with serum from an HIV-1-infected individual. The mutant designation is shown above each lane, and the positions of the viral bands are indicated to the left.

assembles virions and processes the Gag precursor protein in a manner similar to that of the wild type, in contrast to previously described Pol$^-$ vectors (7). It thus provides distinct advantages for glycoprotein incorporation studies. The pFN vector containing either the wild-type or the mutant *env* genes was transfected into COS-1 cells, and at 48 h posttransfection, the cells were labeled as described in Materials and Methods. The culture medium from the labeled COS-1 cells was first clarified, layered onto a 25% sucrose cushion, and centrifuged at 100,000 × *g* for 4 h as described in Materials and Methods. The pellet was resuspended in lysis buffer and immunoprecipitated with serum from an HIV-1-infected individual. The wild-type glycoprotein is efficiently incorporated into virions (Fig. 8, lane labeled WT). It is interesting that in contrast to other studies that indicate only cleaved glycoprotein is transported to the cell surface, both cleaved and a small fraction of uncleaved glycoprotein are incorporated into virions. While all of the mutant glycoproteins are incorporated into virions to some extent, it is clear that the amount associated with virions is significantly reduced in each case. The least amount of glycoprotein is seen for the Δ19 viral pellet, consistent with the degradation of the precursor and reduced surface expression of this glycoprotein described above (Fig. 8, lane labeled Δ19). These results show that even when the cytoplasmic domain is truncated by only 6 amino acids, the amount of virion-associated glycoprotein is significantly reduced (Fig. 8, lane labeled Δ6).

DISCUSSION

Using site-directed mutagenesis, we have constructed a series of point mutations that result in the insertion of premature stop codons throughout the cytoplasmic domain of the HIV-1 glycoprotein. Similar mutations were made in two different *env* genes to reduce the possibility that the effects of mutations would be isolate dependent. The mutations resulted in the deletion of 6 to 192 amino acids from the carboxy terminus of gp41, and their effects on glycoprotein synthesis, processing, and function were determined.

In other viral systems, deletion of the cytoplasmic domain of the viral glycoprotein has been reported to have various effects. For Rous sarcoma virus, deletion of the cytoplasmic domain had no effect on synthesis or transport of the glycoprotein and the mutant virus retained the ability to infect cells (33). In contrast, when deletions were made in the cytoplasmic domain of vesicular stomatitis virus G protein, the transport of the glycoprotein was affected (34). In the studies described here, for most of the mutants, truncation of the cytoplasmic domain of gp41 does not detectably alter the synthesis or processing of the glycoprotein. The exceptions are the Δ139 mutation that results in a reduced amount of cell-associated gp120 and the Δ19 mutation that results in a more rapid degradation of the truncated gp160. A mutation in the glycoprotein of Rous sarcoma virus that inserted a charged amino acid into the membrane-spanning domain also resulted in the rapid degradation of the cell-associated glycoprotein (6). The mechanism of degradation in this case may be different from that of Δ19, however, since it resulted from enhanced endocytosis from the plasma membrane and lysosomal proteolysis of the cleaved gp85/gp37 products (6). The degradation of the Δ19 mutant precursor may be related to the unusual propensity of this region to form an amphipathic helix with a high hydrophobic moment (39). In addition, this region of the glycoprotein has been shown to be protected from antibody recognition when expressed in an in vitro transcription-translocation system, suggesting that the C terminus of gp41 may be associated with the lipid bilayer (14). A disruption of such a membrane association while retaining a very long cytoplasmically located domain might trigger the degradative processes observed for this mutant.

Truncation of the cytoplasmic domain of the hemagglutinin glycoprotein of influenza virus resulted in a more fusogenic glycoprotein (36), and we have observed a similar result with the glycoprotein of Mason-Pfizer monkey virus (2). Studies with HIV-2 have demonstrated an increase in fusogenicity on certain cell types with truncation of the gp41 cytoplasmic domain (31), and previous studies of HIV-1 gp41 mutants also suggested that truncation could be accompanied by increased fusion (10, 22). In the study reported here, the Δ109 mutant glycoproteins induced syncytia that were significantly larger than those induced by the wild type (145%) only when they were expressed in the absence of other viral proteins. In the context of the viral genome, this increase in fusogenicity was not observed. The difference in fusion observed for glycoprotein expressed from the two vectors indicates that it may be important to take into account the expression system utilized in any one study. In each case in which an increase in syncytium formation was previously reported, the mutant protein was expressed from a vector in the absence of other HIV proteins (10, 22).

It has been suggested that the cytoplasmic domain of viral glycoproteins may be utilized by the virus as a signal for their incorporation into virions and therefore might provide a mechanism for excluding cellular surface proteins. Studies of other viruses support such a mechanism (30). The concept of glycoprotein-capsid interactions in retroviruses has been supported by the chemical cross-linking of TM and MA proteins in Rous sarcoma virus (13) and the influence of MA mutations on cleavage of the Mason-Pfizer monkey virus

cytoplasmic domain (3). Moreover, in previous studies we have provided evidence that the HIV-1 glycoproteins can direct the assembly and release of virions to the basolateral surface of polarized epithelial cells (32). Thus, an interaction between the cytoplasmic domain and capsid proteins might play an integral role in virus assembly. The data presented here support this concept since significantly less of the truncated viral glycoproteins are found incorporated into virus particles. This is particularly clear when the levels of gp120 and gp160 incorporated into mutant virions are compared with the amount of $p24^{gag}$ protein. Interestingly, while all the mutant glycoproteins are inefficiently incorporated into virions, it is those with truncations longer than 19 amino acids that appear to be most defective in mediating entry of virus into target cells and show the most complete defect in infectivity. Thus, if there is an essential interaction of the wild-type cytoplasmic domain with components of the budding virion, this interaction may also be necessary for virus infectivity. Such a requirement would also help to explain the dichotomy observed between the efficient syncytial induction in CD4-expressing cells where the truncated glycoproteins mediate cell-cell membrane fusion and the lack of infectivity in both classic virus spread (RT) assays and HXBΔenvCAT complementation.

The incorporation of the mutant glycoproteins into virions may be by way of a nonspecific mechanism similar to the pseudotyping of VSV with various glycoproteins. But it seems unlikely that the block to infectivity is at a postfusion/uncoating stage since a variety of foreign glycoproteins, which would not be expected to interact specifically with the capsid, can be incorporated in a biologically active form into HIV-1 virions. Lusso et al. have shown that the amphotropic murine leukemia virus glycoprotein can mediate entry of HIV into CD4-negative human and rodent cells (25). It has also been demonstrated that the human T-cell leukemia virus type I glycoprotein can extend the host range of HIV-1 to epithelial cells (23). Moreover, full-length and truncated SIV glycoproteins can complement the HXBΔenvCAT construct and mediate entry of the HIV-1 capsid into CEMx174 cells with the subsequent production of CAT activity (18). It is an interesting paradox that the truncated HIV-1 glycoprotein when used in parallel in this assay is unable to complement the defective genome. The truncation in the HIV-1 glycoprotein must interfere with some mechanism that is not blocked in the truncated SIV glycoprotein. Experiments are under way to determine whether the truncated HIV-1 glycoproteins can dominantly interfere when expressed with the SIV glycoprotein.

It is clear from these studies that when single nucleotide changes are used to precisely truncate the cytoplasmic domain, small deletions from the C terminus greatly affect infectivity. Deletions of 42 or more amino acids (for BH10 mutants) or of 67 or more amino acids (for HXB2 mutants) from the C terminus of gp41 completely block the ability of mutant virus to infect target cells. These experiments have been performed with two different isolates of HIV-1 and in different cell lines under conditions in which large amounts of virus have been used to infect cells. In addition, coculture experiments (data not shown) and CAT analysis have confirmed these results. The defect in virus infection appears to be quite profound for truncations of more than 42 amino acids since even when large amounts of virus were used for infection or coculture techniques were used, no reversion of the single nucleotide changes was seen. This suggests that there must be a nearly total block to infection because a low level of infection would likely have resulted in the generation and selection of revertant viruses, as has been described by others (41). Previous studies have reported conflicting effects on infectivity when the cytoplasmic domain of gp41 was altered (12, 24, 38). Some of these differences may be due to the fusion of foreign amino acids onto the C terminus of gp41 or differences in the target cells utilized for infection. While we cannot rule out differences in target cells, we report here similar effects on infectivity in both H9 and CEMx174 cells. Infectivity studies have also been performed with SupT1 cells with similar results (data not shown).

In these studies, we have utilized two different clones of HIV-1. However, it should be pointed out that while these two clones differ significantly in their biological properties, they are closely related. Further studies utilizing more divergent isolates of HIV-1, as well as macrophage tropic strains, will be needed to determine whether the results from this study can be further generalized.

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ADDENDUM IN PROOF

Wilk et al. (T. Wilk, T. Pfeiffer, and V. Bosch, Virology **189**:167–177, 1992) recently reported that HIV-1 virions containing glycoproteins with truncations of 144, 126, and 43 amino acids were infectious in MT4 cells. Preliminary experiments in our laboratory indicate that Δ147, Δ19, and Δ6 can replicate in MT4 cells, even though Δ147 is unable to do so in H9, SupT1, and CEMx174 cells. The remaining mutants, Δ139 and Δ67, were noninfectious in all cell types tested. The basis of the novel restricted host range of Δ147 is under investigation.

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