Truncations of the Simian Immunodeficiency Virus Transmembrane Protein Confer Expanded Virus Host Range by Removing a Block to Virus Entry into Cells

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We have investigated how truncation of the cytoplasmic domain of the transmembrane (TM) glycoprotein of simian immunodeficiency virus (SIV) modulates the host range of this virus. Termination codons were introduced into the *env* gene of SIV_{mac}239 which resulted in the truncation of the transmembrane protein from a wild-type 354 amino acids (TM354) to 207 (TM207) and 193 (TM193) amino acids. Expression of the wild-type and mutant *env* genes from a simian virus 40-based vector resulted in normal biosynthesis and processing of the glycoproteins to gp130 and gp41 or the truncated TM proteins (gp28 and gp27). When expressed on the surface of COS-1 cells, all three glycoproteins mediated fusion of both CEMX174 and HUT78 cells. Virions containing the wild-type and mutant glycoproteins were capable of efficient replication in macaque peripheral blood lymphocytes and CEMX174 cells; in contrast, only virions that contained TM207 were capable of rapid infection of HUT78 cells. Both truncated glycoproteins were capable of efficiently mediating infection of both CEMX174 and HUT78 cells by an *env*-deficient human immunodeficiency virus. The wild-type SIV glycoprotein, however, was unable to mediate human immunodeficiency virus infection of HUT78 cells when assayed with this system. An analysis of the protein composition of SIV released from infected CEMX174 cells showed that the mutant virions contained significantly higher levels of glycoprotein compared with the wild type. These results demonstrate that truncation of the SIV cytoplasmic domain removes a block at the level of glycoprotein-mediated virus entry into HUT78 cells and points to a role for glycoprotein density in determining virus tropism.

Simian immunodeficiency viruses (SIVs) are a group of retroviruses isolated from various species of Old World primates, including African green monkeys (6, 28), sooty mangabey monkeys (13, 23), macaques (2, 4, 26), and mandrills (33). They have been shown to be genetically related to the human immunodeficiency viruses (HIVs), with greatest nucleotide sequence homology to the HIV-2 group (4, 11, 14, 16). Infection of macaques by SIV has been shown to result in an immunodeficiency disease similar to AIDS in humans (5, 22, 30).

SIVs have a morphology similar to that of other retroviruses of the lentivirus family. They possess an electron-dense, conically shaped capsid surrounded by a host cell-derived lipid bilayer into which the viral envelope glycoproteins are inserted. The envelope glycoprotein complex of SIV consists of two subunits, the surface (SU) protein (gp130) and the transmembrane (TM) protein (gp41). The SU protein is involved in binding the CD4 receptor, as do the SU proteins of HIV-1 and HIV-2, on susceptible target cells (18). The TM protein anchors the glycoprotein complex in the viral membrane and appears to play a complex role in mediating fusion of the virus and cell membranes. In contrast to the TM proteins of the nonlentiviruses, the TM protein of SIV, like those of HIV-1 and HIV-2, includes a long cytoplasmic domain of over 150 amino acids, the precise function of which is unknown. It is interesting to note, however, that amplification of various SIV strains in human cell lines has been reported to result in carboxy-terminal truncation of the transmembrane glycopro-

teins due to the creation of in-frame termination codons, resulting in short cytoplasmic domains (4, 12, 16).

SIV_{mac}239 is a pathogenic molecular clone of SIV that encodes a TM protein of 41 kDa with an intact cytoplasmic domain. Virus derived from this molecular clone efficiently infects macaque peripheral blood lymphocytes (PBL), but infection of the human T-cell line HUT78 results in low virus production. Continued passage of this virus in HUT78 cells has resulted in the appearance of a virus encoding a 28-kDa TM protein with a truncated cytoplasmic domain. The truncation of the cytoplasmic domain resulted from the spontaneous substitution of termination codons for glutamine codons 17 to 22 amino acids from the predicted inner membrane boundary. This occurred coincident with the emergence of a virus which replicated with much faster kinetics in HUT78 cells (19). Hence, natural truncation of the TM protein appeared to result in an expanded host range for that virus. It should be noted that no selection for the truncated form occurred upon passage of the virus in stimulated macaque lymphocytes and that the selection occurred only in human-derived T-cell lines (19). Moreover, infection of a macaque with a virus population containing truncated TM proteins resulted in the rapid reversion to virus encoding a full-length TM protein (19).

Molecular clones of two other SIV_{mac} isolates, SIV_{mac}251 and SIV_{mac}142, and of an isolate from the African green monkey have also been shown to encode truncated TM proteins (12, 16, 17, 21, 27). Chakrabarti et al. (3) have examined the functionality of the region of the TM protein C terminal to the truncation in SIV_{mac}142. Mutations in the nonexpressed region indicated that its translation was not required for virus replication in HUT78 cells. Additionally, the premature stop codon was mutagenized to a glutamine,

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and deletions or additional termination codons were introduced 3' of the original stop codon. These modifications abolished infectivity in HUT78 cells (3). Thus, the position of the stop codon in the TM cytoplasmic domain appeared to be critical for maintaining virus infectivity in this cell line.

Similar truncations of the TM protein have been demonstrated to alter the host range of various strains of SIV_{mac} (17, 19). We have investigated the mechanism by which truncation of the SIV TM protein conferred the expanded host range. By using site-directed mutagenesis, we have recreated a naturally occurring truncation (TM207) reported by Kodama et al. (19) and introduced an additional truncation (TM193) which removed the entire predicted cytoplasmic domain, in order to determine whether the residual C-terminal residues were critical for SIV infectivity. We have confirmed that truncation of the TM protein alone to 207 amino acids does confer an expanded host range. Moreover, we have demonstrated that the block in wild-type infection occurred at the level of virus entry. Because mutations that removed the entire cytoplasmic domain of the TM protein resulted in virus that replicated slowly in HUT78 cells, we conclude that the naturally selected truncations of the SIV TM protein confer on this virus a novel growth advantage that alternate truncations do not.

MATERIALS AND METHODS

Cell culture. COS-1 cells were obtained from the American Type Culture Collection (Rockville, Md.). HeLa-T4, CEMX174, C8166, HUT78, 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), Peter Cresswell (CEMX174), and Robert Gallo (C8166, HUT78, and H9). Macaque PBL were a gift from Patricia N. Fultz (University of Alabama at Birmingham). HeLa-T4 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. CEMX174, C8166, HUT78, and H9 cells were grown in RPMI medium 1640 containing 15% fetal bovine serum. Macaque lymphocytes were separated by Ficoll-Hypaque and stimulated with 10 µg of concanavalin A per ml of RPMI medium 1640 containing 15% fetal calf serum for 72 h at 37°C. Macaque lymphocytes were maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum and 8 U human interleukin-2 per ml.

DNAs and plasmids. The molecular clone of an integrated SIV_{mac}239 genome in phage lambda, a gift from Ronald C. Desrosiers, was digested with *Eco*RI, to excise the integrated genome, and then with *Bam*HI at a unique site within the *gag* gene. The 5' *Eco*RI-*Bam*HI fragment of the SIV genome was ligated into a plasmid containing a neomycin resistance cassette, derived from pSV2Neo (32). This neomycin resistance plasmid containing the 5' segment of the SIV genome is designated pNG (Fig. 1A). The 3' *Eco*RI-*Bam*HI fragment of the SIV genome was ligated into pTZ18R, which had been digested with *Eco*RI and *Bam*HI (pSG2; Fig. 1A). To regenerate a complete SIV genome for transfection into COS-1 cells, both pNG and pSG2 were digested with *Bam*HI and religated at 7.5 ng of DNA per µl of ligation mix.

For transient expression of the SIV envelope glycoproteins, plasmid pSRS was constructed. A *Stu*I fragment (nucleotides 6128 to 10045) from the SIV genome which encompasses the *env*, *tat*, and *rev* genes was cloned into pSR, an expression vector containing the simian virus 40

FIG. 1. (A) Diagrams of pNG and pSG2, subgenomic constructs of the SIV genome which when ligated are used for virus production. pNG is a plasmid which contains the neomycin resistance cassette along with the 5' portion of the SIV genome, to the unique *Bam*HI site. pSG2 is a plasmid containing the 3' portion of the SIV genome from the unique *Bam*HI within the genome through the *Eco*RI in the cellular flanking region. The premature termination codons were engineered into pSG2. For virus production, pNG and pSG2 were digested with *Bam*HI, ligated, and transfected into COS-1 cells. LTR, long terminal repeat. (B) Mutations resulting in truncations of the SIV TM protein. A diagram of the SIV TM protein is shown, with the positions of the terminations marked.

(SV40) late promoter and the Mason-Pfizer monkey virus polyadenylation signals (9).

All plasmid DNAs were propagated in *Escherichia coli* DH-1 cells and purified by using cesium chloride gradients as described previously (24).

Oligonucleotide mutagenesis and DNA manipulation. DNA restriction and modification enzymes were purchased from New England Biolabs (Beverly, Mass.) and were used according to the manufacturer's directions or standard techniques (24). Oligonucleotide-directed mutagenesis was performed with the Altered Sites (Promega, Madison, Wis.) mutagenesis system essentially as described earlier (7). A 1.3-kb fragment of the SIV *env* gene was digested with *Acc*I, and the 5' overhang was filled in with deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase. This fragment was ligated into pSELECT1 at the unique *Sma*I site. Single-stranded DNA was generated from this phagemid vector. To generate the truncated envelope genes, translation termination codons were introduced by using the following oligonucleotides: for the 207-amino-acid TM protein (TM207), 5'-CCCTCTTATTTCTAGCAGACC C-3'; for the 193-amino-acid TM protein (TM193), 5'-GCTA AGTTAAGGTAGGGG-3'; and for a 163-amino-acid TM protein (TM163, which results in a secreted glycoprotein, a negative control in several experiments), 5'-GCTTCTTGGA TATAGTATATACAATATGG-3'. These mutations were engineered back into the expression vectors by using *Cla*I and either *Nhe*I or *Bgl*II restriction sites. All mutants were confirmed by dideoxy sequencing utilizing the Sequenase system (United States Biochemical Corp., Cleveland, Ohio).

Metabolic labeling. For transient expression of the envelope glycoproteins, COS-1 cells were transfected by the DEAE-dextran method with the pSRS constructs. At 48 to 72 h posttransfection, the cells were labeled for 30 min with

375 μ Ci of [3 H]leucine (Dupont NEN, Wilmington, Del.) per ml in leucine-deficient Dulbecco's modified Eagle's medium (GIBCO/BRL). After the 30-min pulse, complete medium was added for an additional 4 h. The cells were lysed in lysis buffer A (1% Triton X-100, 50 mM NaCl, 1% sodium deoxycholate, 25 mM Tris-HCl [pH 8.0]), nuclei were pelleted by low-speed centrifugation, and the lysates were immunoprecipitated as described previously (8) with pooled sera from SIV⁺ macaques, obtained from Ronald C. Desrosiers and Patricia N. Fultz. The immunoprecipitated, labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% polyacrylamide gel).

Fusion assay. To determine the capacity of the wild-type and mutant glycoproteins to mediate fusion of CD4-expressing target cells, two approaches were taken. In the first, COS-1 cells were transfected with the pSRS constructs by using DEAE-dextran. One day posttransfection, the transfected cells were trypsinized and reseeded at 2×10^4 cells per 35-mm-diameter plate and overlaid with 10^6 indicator cells. Indicator cell lines included the human T-cell-derived continuous cell lines CEMX174 and HUT78 as well as HeLa-T4. Cultures were visually observed for syncytium formation beginning at day 2 posttransfection. At 48 h posttransfection, HeLa-T4 cells were fixed and stained as previously described (8).

In the second approach, we assayed whether the glycoproteins were capable of cell-cell fusion when expressed directly on HeLa-T4 and HUT78 cells. In these experiments, cells were transfected with the pSRS constructs by using DEAE-dextran. At 48 h posttransfection, HeLa-T4 cells were fixed and stained as previously described (8). The HUT78 cultures were monitored for syncytium formation beginning at day 2 posttransfection.

In some experiments, a wild-type HIV-1 (HXB2) *env*-expressing plasmid pSRHS (8) was used as a positive control.

Infectivity assay. Plasmids pNG1 and pSG2 (Fig. 1), containing the 5' and 3' halves of the SIV genome (the latter containing either a wild-type or mutant *env* gene), were digested with *Bam*HI (New England Biolabs). The digested plasmids were ligated with T4 DNA ligase (New England Biolabs) as described above, and the ligation mix was used to transfect COS-1 cells by the DEAE-dextran method. At 48 h posttransfection, supernatants were collected and analyzed for core antigen (p27) content (SIV Core Antigen Assay Kit; Coulter Immunology, Hialeah, Fla.). A total of 10^6 CEMX174 or HUT78 cells were resuspended in 1 ml of medium containing equal virus amounts, as determined by core antigen assays. After 2 h at 37°C, the cells were transferred to T25 flasks and the volume of the medium was adjusted to 10 ml. The cultures were assayed for reverse transcriptase (RT) activity and split 1:10 at the time points indicated on the graphs (approximately 3-day intervals). CEMX174 infections were fed 10^6 additional uninfected cells each time the cultures were split.

For infection of macaque PBL, virus produced from COS-1 cells (as described above) was grown in CEMX174 cells for 7 to 10 days. Equivalent virus amounts, as determined by RT activity in the culture supernatant, were incubated in a 1-ml volume with cells for 2 h at 37°C. The cells were pelleted, and the virus-containing medium was replaced with 10 ml of RPMI 1640 supplemented with fetal bovine serum and interleukin-2. Eighty percent of the macaque PBL culture medium was changed at 5-day intervals. The cultures were assayed for RT activity daily. For

extended studies, additional concanavalin A-stimulated PBL were added to each culture at 10-day intervals (long-term infectivity data not shown).

RT assays. RT activity from infected culture supernatant was assayed as previously described (7).

Virus incorporation of wild-type and truncated glycoproteins. CEMX174 cells infected with SIV containing either wild-type or truncated TM proteins were pulse-labeled for 40 min with [35 S]cysteine/methionine (Expre 35 S 35 S; NEN) in cysteine- and methionine-deficient medium. Cells were then chased for 6 h in RPMI containing 10% fetal bovine serum. A 9-ml aliquot of the culture supernatant was clarified by low-speed centrifugation and then filtered through a 0.45- μ m-pore-size filter, layered onto a 1.5-ml serum cushion, and centrifuged at 25,000 rpm for 2 h in an SW41 rotor. The viral pellets were resuspended in lysis buffer and immunoprecipitated as described above. To assess the total amount of viral protein present in the culture supernatant, a 1-ml sample was immunoprecipitated directly prior to pelleting. Immunoprecipitates were analyzed by SDS-PAGE (10% polyacrylamide gel).

pHXB Δ envCAT complementation assay. To assess the ability of wild-type and mutant SIV glycoproteins to allow entry of virus in a single-step infection, HIV complementation assays were used. These were performed as described previously (15) with the exception that the SIV envelope glycoproteins were used to complement an HIV-1 genome deficient for envelope glycoprotein expression. COS-1 cells were cotransfected with pHXB Δ envCAT, a gift from Joseph Sodroski, and pSRS containing either the wild-type or mutant SIV *env* gene, or in some cases pSRHS containing wild-type and mutant HIV *env* genes (8). At approximately 60 h posttransfection, the culture medium was collected, filtered, and assayed for RT activity. A total of 10^7 CEMX174 or HUT78 cells were infected as described above with equivalent amounts of complemented HIV, as determined by RT assays. At approximately 60 h postinfection, the cells were lysed by repeated freeze-thawing, and a portion of the cell lysate was assayed for chloramphenicol acetyltransferase (CAT) activity (31). Each of the cell lysates was incubated for 2 h with [14 C]chloramphenicol and acetyl coenzyme A, after which the reactions were stopped and the products were subjected to thin-layer chromatography. CAT activity was measured as the percent conversion of [14 C] chloramphenicol to the monoacetylated form, as measured with the AMBIS radioanalytical imaging system.

RESULTS

Construction of plasmids encoding truncated SIV glycoproteins. To examine the role of cytoplasmic domain truncations in the altered biologic properties of SIV glycoproteins, two site-directed mutations which created premature termination codons in the envelope open reading frame were made. These mutations resulted in transmembrane glycoproteins of 207 and 193 amino acids (Fig. 1B). The designed mutations do not affect the overlapping *tat* or *rev* coding regions. The mutated *env* gene sequences were then cloned into pSRS, an SV40-based expression vector containing the entire SIV *env* gene, for studies of mutant protein biosynthesis and function. To analyze the effects of the mutations on virus replication and host range, they were engineered into pSG2. Ligation of this plasmid, which contains the 3' two-thirds of the SIV genome, to pNG, which contains the remainder of the SIV genome (Fig. 1), and transfection into COS-1 cells results in production of infectious virus.

FIG. 2. Synthesis and processing of wild-type and mutant glycoproteins. COS-1 cells were transfected with pSRS containing either the wild-type or mutant *env* gene. At 60 h posttransfection, the cells were pulse-labeled with [3 H]leucine for 30 min, subjected to a 4-h chase, and then lysed. The cell lysates were immunoprecipitated with serum from SIV $^{+}$ macaques. Molecular weights of the TM proteins are indicated.

Wild-type and truncated glycoproteins of SIV are synthesized and processed normally when expressed from an SV40 expression vector. To examine the synthesis and processing of these truncated glycoproteins, COS-1 cells were transfected with pSRS vectors containing mutant and wild-type envelope genes. At 60 h posttransfection, the cells were metabolically labeled with [3 H]leucine. After a 30-min pulse followed by a 4-h chase, the cells were lysed and the cell lysates were immunoprecipitated with serum from an SIV-infected monkey. Immunoprecipitates were analyzed by SDS-PAGE and subjected to autoradiography. As shown in Fig. 2, the wild-type and mutant glycoprotein precursors (gp160) were expressed at similar levels and were processed normally during the chase, as evidenced by the production of the SU protein (gp130) and either full-length (gp41) or truncated (gp28 and gp27) TM proteins (Fig. 2). While equivalent amounts of the TM163 glycoprotein were observed in pulse-labeled cells, processed products were not found associated with the cell lysates after the 4-h chase; however, immunoprecipitation of the chase culture medium demonstrated that levels of cleaved products, similar to those seen associated with the cell for the wild-type gene, were released from the cell (data not shown).

SV40-expressed wild-type and mutant glycoproteins are fusogenic in a syncytium-forming assay. Because one assay of a glycoprotein's ability to bind to the CD4 receptor and initiate virus entry is cell-cell fusion, we sought to determine whether the expressed glycoproteins would produce syncytia with various CD4-expressing cell lines. COS-1 cells transfected with the glycoprotein expression vectors were overlaid with various T-cell lines, including HUT78 and CEMX174, and observed daily for syncytium formation. As can be seen in Fig. 3, all three glycoproteins (TM354, TM207, and TM193) were capable of inducing cell-cell fusion with both CEMX174 and HUT78 cells. As a negative control, the pSRS.TM163 construct, which expressed a secreted variant of the SIV glycoprotein, was used. No syncytia were detected in either HUT78 or CEMX174 cells following addition to COS-1 cells expressing this construct. Additional syncytium assays have shown that COS-1-expressed glycoproteins (TM354, TM207, and TM193) are fusogenic with a wide variety of additional T-cell lines, including C8166 and H9 (data not shown).

In these experiments, the wild-type glycoprotein was clearly capable of inducing fusion of COS-1 cells with HUT78 cells, a cell line in which virions containing the same glycoprotein replicate minimally (19). Therefore, to rule out

a possible role for the COS-1 cell membrane in the fusion process, HUT78 cells were transfected directly, by the DEAE-dextran method, with the pSRS expression plasmids. As can be seen in Fig. 3, equivalent (in both size and number) levels of syncytium formation were noted in cultures expressing TM354, TM207, and TM193.

HeLa-T4 cells have been used in studies to determine the syncytium-forming capacity of mutant HIV-1 glycoproteins (7, 8). To determine whether the wild-type or mutant SIV proteins could induce syncytium formation in these cells, both direct transfection and cocultivation experiments were performed (Table 1). Following direct transfection, no cell-cell fusion was observed for any of the SIV constructs in HeLa-T4 cells; in contrast, transfection of the HIV-1-expressing construct, pSRHS, resulted in numerous multinucleated cells with more than 20 nuclei, as we have described previously (8). To rule out the possibility that transport or processing of the mutant or wild-type SIV glycoproteins might be impaired in HeLa-T4 cells, COS-1 cells expressing wild-type or mutant SIV glycoproteins were mixed with HeLa-T4 cells. These cocultivation experiments resulted in no syncytium formation with any of the SIV glycoprotein-expressing cells; cells expressing the HIV-1 glycoprotein, in contrast, yielded large syncytia and provided a positive control in these experiments (Table 1). Thus, while the SIV glycoprotein can induce syncytia in a variety of human T-cell lines, it is unable to mediate fusion of a human epithelial cell line that is readily fused by HIV-1.

Infectivity of virions containing truncations in the cytoplasmic domain of their transmembrane proteins on human T-cell lines. When COS-1 cells were transfected with the ligated pNG and pSG2 plasmids, virion production was detected in wild-type and mutant cultures by a core antigen capture assay. Transfection of pNG alone into COS-1 cells resulted in no detectable core antigen. Supernatants from COS-1 cells transfected with the different ligated viral constructs were assayed for core antigen content, and equivalent amounts of virus, as determined by this assay, were used to infect CEMX174 and HUT78 cells.

As shown in Fig. 4A, in CEMX174 cultures, a rapid spread of virus infection was observed for virions containing the wild-type or either of the membrane-anchored mutant glycoproteins. Infection of C8166 cells results in similar kinetics of infection for virions containing TM354, TM207, and TM193 (data not shown). In contrast, in HUT78 cells, only virions expressing the TM207 glycoprotein exhibited the same rapid spread of infection (Fig. 4B). Repeated experiments showed a marked delay in the appearance of RT activity in the medium of HUT78 cells infected with wild-type virions, consistent with the previous report of Kodama et al. (19). Interestingly, virions containing a glycoprotein complex with the 193-amino-acid-long TM protein showed a 10-day delay in the appearance of RT activity. It was unlikely that this virus was undergoing a conversion to the naturally occurring truncation during this short delay, since reversion to wild type followed by conversion to the natural truncation would require two independent mutations. Nevertheless, to determine whether virions containing the 193-amino-acid TM protein had reverted to a rapid phenotype, HUT78 cells were infected with TM207 and TM193 virus derived from HUT78 cells on days 21 and 35 postinfection. A delay in the spread of infection by TM193-containing virions similar to that shown in Fig. 4B was observed in these reinfections of HUT78 cells (Fig. 4C), although in these experiments with high-titer virus, RT activity for both TM207 and TM193 appeared more rapidly; thus, truncation

FIG. 3. Fusion assay of wild-type and truncated glycoproteins expressed from pSRS. COS-1 cells were transfected with pSRS containing either the wild-type or mutant *env* gene. One day posttransfection, the transfected cells were split and overlaid with either CEMX174 cells (COS-1/CEMX174) or HUT78 cells (COS-1/HUT78). Alternatively, HUT78 cells were transfected directly with the envelope expression plasmids and monitored for syncytium formation (HUT78 direct).

of the TM protein of SIV to 193 amino acids results in virus with a delayed but infectious phenotype. These observations were supported by preliminary polymerase chain reaction studies of infected HUT78 cell DNA at day 24 postinfection, in which six of six polymerase chain reaction clones (nucleotides 8308 to 9520) retained the TM193 mutation and demonstrated no additional changes (data not shown).

TABLE 1. Syncytium formation after transfection of HeLa-T4 cells or transfection of COS-1 cells cocultivated with HeLa-T4 cells

Mutant	Avg no. of nuclei/syncytium ^a	
	HeLa-T4 direct	COS-1/HeLa-T4 cocultivation
HIV wild type (pSRHS)	21.2	35.8
SIV TM354	<2	<2
SIV TM207	<2	<2
SIV TM193	<2	<2
SIV TM163	<2	<2

^a The nuclei of 10 syncytia were counted for each construct. More than 200 syncytia per plate were present in pSRHS-expressing cultures. Values of <2 represent cultures in which no syncytia were detectable.

When virus grown to high titers in CEMX174 cells was used to infect HUT78 cells, a similar delay in the appearance of RT activity was seen for virions containing the TM354 glycoprotein (Fig. 5A). It therefore appears that there is no selection for the truncated TM protein when these virions are passaged in CEMX174 cells. Interestingly, when the high-titered TM193 virus was used to infect HUT78 cells, the delay in appearance of infectious virus was shortened to only 3 to 4 days.

Virions carrying TM354, TM207, or TM193 are infectious for macaque PBL. Previous studies had shown that HUT78 cell-derived SIV_{mac239} stocks, containing virions with both wild-type and truncated TM proteins, were capable of growth in macaque PBL (19). Since in the latter experiments, selection for the wild-type virus occurred over time, we sought to determine whether virus containing the TM207 truncated glycoprotein could replicate in macaque PBL in the absence of virus containing the wild-type glycoprotein. We also determined whether the absence of the putative cytoplasmic domain in TM193-containing virions limited virus replication in macaque PBL.

High-titer virus stocks grown in CEMX174 cells were used to infect concanavalin A-stimulated macaque PBL. As can

FIG. 4. Infectivity assay of SIV TM mutants on CEMX174 cells or HUT78 cells. COS-1-produced virions containing either the wild-type or mutant *env* gene were used to infect either CEMX174 (A) or HUT78 (B) cells as described in Materials and Methods. Additionally, TM207 and TM193 containing virions collected from HUT78 infections at day 35 were used to infect HUT78 cells (C). At the times indicated, aliquots of the infected culture supernatants were taken and assayed for RT activity.

be seen in Fig. 5B, all three virus stocks were capable of rapid infection of macaque PBL, as demonstrated by the rapid increase in RT activity in the culture supernatants. Thus, the naturally truncated Env glycoprotein can confer infectivity in the absence of the wild-type protein. Moreover, as demonstrated with TM193, it appears that the

FIG. 5. Infectivity of high-titer viruses on HUT78 cells or macaque PBL. Virus grown to high titer on CEMX174 cells was used to infect HUT78 cells (A) or macaque PBL (B). At the times indicated, culture medium was collected and RT activity was assayed.

cytoplasmic domain of the TM protein is not essential for infection of PBL. Cultures infected with wild-type virions and virions containing either TM207 and TM193 maintained high-titer virus production for up to 30 days (data not shown). The question of whether long-term culture of these viruses in macaque PBL results in reversion to wild type is currently being addressed.

Wild-type glycoprotein exhibits an entry level defect for infection of HUT78 cells. To investigate whether a defect at the level of virus entry was responsible for the lack of infectivity of wild-type SIV and for the delayed infection with TM193 virus in HUT78 cells, a rapid complementation system first described by Helseth et al. (15) was used. For these experiments, the *env*-deficient HIV-1 construct pHXB Δ envCAT (designated pCAT in Fig. 6), which contains the CAT gene, was cotransfected into COS-1 cells with the SIV and HIV-1 *env* expression plasmids. Coexpression of the two plasmids resulted in the production of pseudotyped, CAT-encoding particles which were used to infect various cell lines. The efficiency of infection was calculated from the level of CAT produced in the infected cells. Consistent with infection data presented above, SIV TM354, TM207, and TM193 *env*-expressing plasmids were all capable of efficiently complementing the defective HIV-1 genome when infectivity was assayed on CEMX174 cells. Indeed, the SIV Env-pseudotyped virus was able to infect these cells

FIG. 6. Complementation of HIV-1 infection with SIV wild-type or mutant glycoproteins. COS-1 cells were cotransfected with pSRS, an SV40-based expression vector containing either the wild-type or mutant SIV *env* gene, and pHBΔenvCAT. As controls, pSRS, the same vector containing either wild-type or mutant HIV-1 *env* gene, and pHBΔenvCAT were cotransfected into COS-1 cells. Supernatant was collected at 60 h and assayed for RT activity. Equal virus amounts, as determined by RT activity, were used to infect either CEMX174 cells or HUT78 cells. At 60 h postinfection, the cells were lysed by repeated freeze-thawing, and the cellular lysate was assayed for CAT activity.

more efficiently than was the HIV Env-containing virus. In contrast, the same wild-type SIV Env-containing HIV stock that could infect CEMX174 cells was almost completely excluded from HUT78 cells, yielding background levels of CAT activity similar to those of the nonpseudotyped HXBΔenvCAT virions. Only those virions containing the wild-type HIV glycoprotein or the TM207 and TM193 truncated SIV glycoproteins were able to efficiently infect HUT78 cells. Interestingly, the HIV Δ139 mutant glycoprotein, in which the cytoplasmic domain has been truncated by substitution of a stop codon for a glutamine analogous to that substituted in SIV.TM207, was unable to complement the defective HIV provirus in either cell type. This result is consistent with our previous studies of HIV cytoplasmic domain truncation mutants (8).

Since SIV TM193 and TM207 Env-containing HIV-1 virions were almost equally infectious on HUT78 cells, the delayed infection of HUT78 cells by virions containing TM193 does not appear to be due to an entry-level defect. In contrast, the lack of complementation by the SIV TM354 glycoprotein on HUT78 cells suggests that there is a block at the level of virus entry.

The relative amounts of CAT activity in cells infected with HIV virions complemented by the wild-type glycoprotein versus those complemented by the truncated mutant glycoproteins were reproducible in more than four independent experiments; Fig. 6 shows a representative sample.

Incorporation of wild-type and truncated glycoproteins into virions. To determine the relative levels of glycoprotein incorporation into the wild-type and mutant virions, we pulse-labeled infected CEMX174 cells (permissive for both wild-type and mutant virus). The culture medium was collected after 6 h, clarified by low-speed centrifugation, and filtered through a 0.45-μm-pore-size filter. These supernatants were then layered onto a serum cushion, and the virus was pelleted as described in Materials and Methods. The viral pellets, resuspended in lysis buffer, were immunoprecipitated with serum from an SIV-seropositive macaque. As

FIG. 7. Reduction of incorporation of wild-type glycoprotein into virus relative to incorporation of truncated glycoproteins. Cells infected with virions containing wild-type and truncated glycoproteins were pulse-labeled with [³⁵S]cysteine/methionine and then chased for 6 h in complete medium. Virus, pelleted from the filtered supernatants, was immunoprecipitated and analyzed by SDS-PAGE (10% polyacrylamide gel). Filtered supernatant was also analyzed to determine overall levels of viral proteins present. The position of the SU component of the glycoprotein complex is denoted by gp130. The position of the major capsid protein is denoted by p27.

demonstrated in Fig. 7, while equivalent levels of p27 (major capsid [CA] protein) were released into the culture medium for each of the viruses, the levels of glycoprotein incorporation differed markedly (Fig. 7, Viral Pellet). The gp130 of wild-type SIV_{mac}239 (TM354) was present in virions at a significantly reduced level relative to that of viruses encoding the truncated TM glycoproteins. Since it was possible that gp130 in the wild-type might have a less stable association with its TM protein and was released during pelleting through the serum cushion, the overall level of viral proteins present in the culture supernatant prior to pelleting was analyzed (Fig. 7, Supernatant). Again, the level of wild-type glycoprotein, relative to that of p27, was significantly lower than the levels of the truncated glycoproteins. Thus, the lack of virion-associated gp130 in wild-type virions was not due to shedding during purification. Instead, it appears that truncation of the SIV TM protein results in increased incorporation of the glycoprotein into virions.

DISCUSSION

SIVs isolated from macaques and various other nonhuman primates encode an *env* gene product with a long cytoplasmic domain that is similar in length to and has sequence homology with the long cytoplasmic domain of HIV-1. In contrast to HIV-1, only a single isolate of which has been reported to express a truncated TM protein (29), several SIVs which were cloned after propagation in human T-cell lines have been shown to encode TM proteins truncated in the cytoplasmic domain (4, 12, 16). These truncations appear to be a result of virus propagation in human cell lines, since analyses of viruses recovered from infected monkeys indicated the presence of nontruncated cytoplasmic domains (17, 19). Moreover, Kodama et al. (19) showed that propagation of the SIV_{mac}239 virus in certain human cell lines resulted in the selection of viruses that grew much more

efficiently in the human cells than did the wild-type virus and now encoded a truncated TM protein.

The mechanism by which these truncations facilitated infection of human cells was unknown. By site-directed mutagenesis, we have re-created the previously reported premature termination signal which results in a truncated TM protein in SIV_{mac}239 and have confirmed that truncation of the TM protein can by itself enable efficient virus replication in HUT78 cells. Studies with SIV_{mac}142 (3) and SIV_{mac}251 (BK28) (17) have also demonstrated that truncation of the TM protein results in an altered cell tropism in the context of virus. To elucidate the mechanism by which truncation of the SIV_{mac}239 TM protein enhanced virus replication in selected human cell lines, we have examined various parameters of virus infection, including cell-cell fusion, virus entry, and viral replication.

Expression of the HIV or SIV glycoprotein on the surface of CD4 receptor-positive cells can result in the formation of multinucleated cells. Such an assay thus provides a measure of the ability of a glycoprotein to be transported to the cell surface, bind in a biologically meaningful manner with its receptor, and catalyze the fusion of apposing cell membranes. In an initial attempt to determine whether the wild-type SIV glycoprotein, which could not mediate infection of HUT78 cells, was defective in productively interacting with the receptor molecule on the surface of these cells, we examined the syncytium-forming ability of the wild-type and truncated TM proteins in both permissive and nonpermissive cells. Surprisingly, it was found that following expression from an SV40-based vector system, each of the glycoproteins was capable of inducing cell-cell fusion in both permissive (CEMX174 and C8166 [data not shown]) and nonpermissive (HUT78 and H9 [data not shown]) cell lines. The ability of the wild-type glycoprotein to induce syncytium formation in HUT78 cells was not dependent on it being expressed in COS-1 cells, since fusion was observed in both direct and indirect fusion assays. These results suggested that the wild-type SIV glycoprotein was capable of efficiently interacting with the CD4 receptor on HUT78 cells and of catalyzing membrane fusion, even though these cells allow minimal replication of virions containing a wild-type glycoprotein.

Truncations of the transmembrane glycoproteins of HIV-2, similar to those described for SIV, have been observed (1, 10, 14, 20). HIV-2/ST is a noncytopathic HIV-2 strain which encodes a TM protein with a cytoplasmic domain of greater than 150 amino acids. Propagation of HIV-2/ST in SupT1 cells resulted in a more cytopathic strain, designated HIV-2/ST/24.1C#2, which contained truncated TM proteins with cytoplasmic domains retaining only 17 amino acids. Analysis of the envelope glycoproteins of the parental and progeny strains in vaccinia virus expression vectors has demonstrated that the TM protein truncation enhances the cell-cell fusion activity of this glycoprotein complex with SupT1 cells, the cell line on which this virus was selected (25). In contrast, the studies presented here suggest that truncation of the SIV TM protein does not increase its syncytium-forming ability. Our data suggest that increased cell-cell fusion is unlikely to be the mechanism involved in the enhanced replication in HUT78 cells of SIV virions containing truncated TM proteins.

SIV virions released from COS-1 cells following transfection of the wild-type and mutant molecular clones showed a host range restriction on HUT78 cells that paralleled that described previously by others (3, 17, 19). Mutant virions containing the TM207 truncated glycoprotein replicated rap-

idly and without delay, whereas the wild-type virions did not show evidence of replication for more than 3 weeks. These infectivity assays, which use RT activity as a measure of virus spread, require multiple rounds of virus replication to yield detectable RT levels. It was therefore possible that reduced glycoprotein expression or impaired assembly of the wild-type glycoproteins might occur in infected HUT78 cells, resulting in very slow virus spread. To investigate whether in the context of a virion the wild-type glycoprotein was defective in mediating virus entry, we used a single-cycle HIV complementation system. In this assay, wild-type and mutant glycoproteins expressed from an SV40-based vector were used to complement an *env*-defective HIV provirus. HIV virions containing the wild-type (TM354) SIV glycoprotein were more efficient at infecting CEMX174 cells than were virions complemented by the HIV-1 wild-type glycoprotein itself and showed infection levels similar to those of virions containing the truncated TM207 glycoprotein. Thus, the SIV glycoprotein can efficiently complement HIV entry into susceptible target cells. In contrast, the same (wild-type TM354) complemented virus stock showed marginal infectivity on HUT78 cells and in other experiments yielded CAT values indistinguishable from those of the negative controls. HIV particles bearing the SIV.TM207 glycoproteins, as might be predicted from HUT78 replication assays, efficiently entered these cells. These results suggest that in the context of a virus particle, the full-length SIV glycoprotein is unable to mediate fusion or entry of the viral capsid into HUT78 cells, even though the glycoprotein itself is capable of initiating cell-cell fusion in the same cells. We have observed a similar phenomenon in studies of HIV-1, in which truncations of the cytoplasmic domain had no effect on the ability of the protein to induce cell-cell fusion but drastically reduced virus infectivity (8). Indeed, as we show here, HXBΔ*env*CAT virions containing the Δ139 mutant HIV glycoprotein were unable to infect either CEMX174 or HUT78 cells. Our studies with the HIV truncations pointed to a reduced incorporation and stability of the mutant glycoprotein in virions (8), and experiments aimed at quantitating the level of glycoprotein in SIV virions demonstrated that the wild-type SIV glycoprotein is also less efficiently assembled. It is important to note that the decreased level of glycoprotein in the wild-type virions does not appear to affect virus entry into a number of CD4⁺ cells, including CEMX174, C8166, and macaque PBL, but is specific for HUT78 and H9 cells.

The molecular basis for the entry defect of wild-type SIV on HUT78 cells remains unclear. It cannot reflect an intrinsic defect in productively binding the receptor, since the same protein expressed to high levels in these cells is fusogenic. Rather, these results point to a unique requirement of the glycoprotein in the context of a virion that on HUT78 cells prevents the complex series of interactions with CD4 that are necessary for viral membrane-cell membrane fusion. It is possible that in HUT78 cells there is a requirement for a greater density of glycoprotein-receptor interactions, at the point of virus attachment, in order for the viral membrane to fuse with that of the cell. The higher concentration of glycoprotein in the mutant virus particles may be sufficient for this process to occur, whereas that of the wild-type virions may not. The difference in susceptibilities of different cell types might reflect a difference in composition of membrane lipids or cellular membrane proteins which interact with the virus at the level of entry, either during fusion or at a postfusion stage. We are now analyzing

directly whether wild-type virions are capable of fusing with HUT78 cells.

Nucleic acid sequencing of a number of truncated SIV glycoprotein genes selected following growth in human cells showed that the truncation was the result of single nucleotide changes at any of three glutamine codons (residues 208, 209, and 214 of the TM protein) (17, 19). An additional glutamine codon is located at residue 194 of the SIV_{mac}239 TM protein, but there have been no reports of spontaneous truncations occurring by mutation of this CAG codon. Moreover, the 14-amino-acid region between glutamine 194 and glutamine 208 is highly conserved between SIVs of diverse origin. To determine whether this region of the cytoplasmic domain was important for virus replication, we substituted a termination codon (TAG) for the glutamine codon (CAG) at position 194. We have demonstrated that this additional truncation which removed the entire cytoplasmic domain of the SIV TM protein does not interfere with glycoprotein function. Expression of the TM193 mutant glycoprotein in COS-1 cells resulted in cell-cell fusion after mixing with a variety of CD4-bearing cell lines. Moreover, SIV virions containing the TM193 mutation were highly infectious for macaque PBL and CEMX174 cells and retained the ability to infect HUT78 cells. However, virus containing this truncated protein did exhibit a delay in replication in the latter cells. The delay was not as pronounced as that for the wild-type TM354 virus (10 days versus 15 days) and may reflect a decrease in replication kinetics rather than a defect in entry, since HIV complemented with this mutant *env* gene product was able to efficiently infect HUT78 cells. Nevertheless, the delayed infectivity in HUT78 cells of SIV encoding this glycoprotein may be sufficient to exclude its selection during the growth of wild-type SIV in HUT78 cells. Chakrabarti and coworkers have demonstrated previously that insertion of stop codons C terminal to the spontaneous truncations abolished the expanded host range (3). These observations together with the ones presented here suggest that the insertion of stop codons at positions 208 to 214 in TM confer a unique change in the glycoprotein that allows infection of certain human cell lines.

The TM193 and TM207 mutant viruses not only replicate on CEMX174 and HUT78 cells but also grow in macaque PBL in culture. Previous studies had suggested that the truncated glycoprotein-containing virus was selected against during growth in macaques or on macaque PBL (17, 19), and SIV_{mac}142, a strain genetically related to SIV_{mac}239 that encodes a truncated TM protein, is incapable of virus replication in macaque PBL (3). Since we have shown that virions containing only truncated TM proteins are capable of PBL replication, it seems likely that other modifications have occurred to SIV_{mac}142 which limit its ability to replicate in macaque PBL. Nevertheless, it remains to be determined what role the cytoplasmic domain of SIV plays during infection in vivo and whether in the monkey a stronger selection mechanism exists to allow outgrowth of wild-type virus. The mutant genomes described in this report combined with deletions in this region should provide important tools for such studies.

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