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Specific Targeting to CD4$^+$ Cells of Recombinant Vesicular Stomatitis Viruses Encoding Human Immunodeficiency Virus Envelope Proteins

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We generated replication-competent, recombinant vesicular stomatitis viruses (VSVs) expressing the human immunodeficiency virus (HIV) envelope protein or an HIV-VSV chimeric envelope protein in which the cytoplasmic domain of the HIV envelope protein was replaced with that from the VSV glycoprotein (G). These recombinants were generated with HIV type 1 (HIV-1) envelopes from both laboratory and primary isolates of HIV-1. The replication-competent recombinant viruses were stable and expressed the foreign proteins at high levels from extra transcription units in VSV. The foreign proteins were processed appropriately and transported to the cell surface. The incorporation of HIV gp120 into VSV particles was demonstrated biochemically only for the construct expressing the chimeric envelopes containing the VSV G cytoplasmic domain. The incorporation of the chimeric HIV envelope protein into the membrane of the recombinant VSV was also demonstrated by electron microscopy with gold-conjugated antibodies. To determine whether specific infection of CD4-positive cells could be demonstrated for these recombinants, we neutralized VSV infectivity due to VSV glycoprotein with anti-VSV serum. The neutralized recombinants expressing the chimeric envelope were able to infect only HeLa cells expressing CD4, and this CD4-specific infectivity was neutralized with anti-HIV serum. This assay also detected a 100-fold-lower titer of CD4-specific infectivity for the VSV recombinant expressing the wild-type HIV envelope. Our results illustrate that it is possible to express functional HIV envelopes from the VSV genome and target the recombinant virus to an alternative receptor. The recombinants may also prove useful as HIV vaccines.

Vesicular stomatitis virus (VSV) is a membrane-enveloped virus that is the prototype of the *Rhabdoviridae*, a large family of viruses that infect vertebrates, invertebrates, and plants. VSV causes a self-limiting disease in livestock and is nonpathogenic or mildly pathogenic in humans (reviewed in reference 28). VSV has been used extensively as a molecular and cell biology tool for many years. Its popularity results from the simplicity of its structure and its very rapid growth to extremely high titers in most mammalian and other animal cells. VSV has a single, negative-stranded RNA genome and carries an RNA-dependent RNA polymerase that transcribes the 11,161-nucleotide genomic RNA to generate subgenomic mRNAs. The subgenomic mRNAs encode the following five structural proteins: the nucleocapsid protein (N), which encases the genome tightly; two polymerase subunits, termed L and P; an internal matrix protein (M); and a single transmembrane glycoprotein (G), responsible for binding virus to cells and for membrane fusion (28). Although neither the full-length genome nor antigenome RNAs of rhabdoviruses are infectious, methods are available that allow the assembly of viral nucleocapsids in cells and recovery of complete infectious rabies virus (27) or VSV (13, 30) from DNA, thus enabling genetic engineering of recombinant rhabdoviruses. Similar systems have also been used to recover Sendai (11), measles (22), and respiratory syncytial (3) viruses, three larger nonsegmented negative-strand RNA viruses from the family *Paramyxoviridae.*

Because VSV grows so well in a wide variety of cells, its potential as a vector for the expression of foreign genes has previously been investigated. The insertion of an extra transcription unit between the VSV G and L genes was found to support high-level, stable expression of an extra mRNA and protein (26). In a second study, it was found that both cellular and viral membrane glycoproteins could be expressed at high levels from recombinant VSVs (25). These foreign membrane glycoproteins were incorporated at high levels into the VSV envelope along with VSV G protein and did not require specific signals for incorporation.

Such efficient incorporation of foreign membrane proteins into VSV was unexpected based on earlier work from our laboratory showing that the human immunodeficiency virus type 1 (HIV-1) Env protein expressed using a vaccinia virus-T7 system was not detectably incorporated into virions of a VSV G mutant in which G is not transported to the cell surface at a nonpermissive temperature. The incorporation of HIV envelope was obtained only with an HIV-VSV chimeric protein in which the HIV Env protein tail was replaced with the VSV G tail (21). Similar results have been obtained with a rabies virus complementation system where chimeric HIV Env-rabies virus G proteins were incorporated into defective rabies virions that were deficient in the rabies virus spike glycoprotein (16). The requirement for the cytoplasmic tail was also surprising since VSV (HIV) pseudotypes containing the wild-type HIV envelope had been described previously (5). These apparent discrepancies led us to analyze further the requirement for the VSV G tail in VSV recombinants expressing the HIV Env protein and wild-type VSV G. We show here that even in the presence of wild-type VSV G, only the chimeric HIV-VSV envelope protein was incorporated into VSV particles at levels detectable biochemically. These recombinants, after neutral-

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ization with antibodies to VSV, could specifically infect $CD4^+$ cells. Similar assays with VSV recombinants expressing wild-type envelope protein revealed a much lower level of CD4-specific infectivity, indicating that the requirement for the G tail is not absolute. The expression of functional HIV envelope glycoprotein from VSV recombinants and its incorporation into VSV particles raise the possibility that live or even inactivated VSV recombinants might be useful as HIV vaccines.

MATERIALS AND METHODS

Plasmid construction. PCRs (24) were performed with VENT polymerase (New England BioLabs) to minimize introduction of sequence errors. The HIV gp160 gene was amplified by PCR from plasmid pNL4-3 (1). The forward primer was 5′-GGACGCGTCTCGAGATTATGAGAGTGAAGGAG, and the reverse primer was 5′-GCTCTAGACCCATCTTATAGCAAAATCC. The primers contained the indicated *Xho*I and *Xba*I sites (underlined). The PCR product was cloned through the *Xho*I and *Xba*I sites into pVSV-XN1 (26) that had been digested with *Xho*I and *Nhe*I. The resulting plasmid was designated pVSV-gp160. The gp160G gene, encoding the chimeric protein that contains the extracellular and transmembrane domains of the HIV gp160 molecule fused to the cytoplasmic tail portion of the VSV G molecule, was amplified by PCR from plasmid pEnvG709 (21). The forward primer was that described above, and the reverse primer was 5′-ACGTACGTGCTAGCTTACTTTCCAAGTCGGTTC, containing the underlined *Nhe*I site. The PCR product was digested with *Xho*I and *Nhe*I and cloned into the *Xho*I and *Nhe*I sites of pVSV-XN1. The resulting plasmid was called pVSV-gp160G.

The full-length 89.6 gp160 gene was amplified by PCR from a subclone containing the *env* region of the 89.6 infectious clone (7). The forward primer was 5′-AACCTTGGAACCCGGGGTCGACACAATGAGAGTGAAGGAGAT CAG, containing the underlined *Sal*I site. The reverse primer was 5′-AACCTT GGAAGCGGCCGCGCTAGCCTTATAGCAAAGCCCTTTCC, containing the underlined *Nhe*I site. Because the coding sequence of the 89.6 gp160 gene contains an *Xho*I site, the PCR primer was designed with a *Sal*I site at the 5′ end, which is compatible for cloning into the *Xho*I site of the full-length pVSV-XN1 vector. The PCR product was digested with *Sal*I and *Nhe*I and cloned into the *Xho*I and *Nhe*I sites of pVSV-XN1. The resulting plasmid was designated pVSV-89.6gp160.

The 89.6gp160G construct was prepared in a manner similar to that of the original gp160G construct. PCR was used to amplify the DNA encoding the extracellular and transmembrane domains of the 89.6 gp160 protein and the DNA encoding the cytoplasmic tail of the VSV G protein. The tail sequence was amplified from plasmid pEnvG709 with forward primer 5′-AACCTTGGAAGG ATCCATCTTTGCATTAAA, containing a *Bam*HI restriction site in the coding region (underlined) and a total of 20 nucleotides of coding sequence in the G gene. The reverse primer was the same as that listed above for the original gp160G clone. The product was purified and digested with *Bam*HI.

DNA encoding the 89.6 gp160 extracellular and transmembrane domains was amplified with forward primer 5′-AACCTTGGAACCCGGGCTCGAGACAA TGAGAGTGAAGGAGATCAG, containing the underlined *Xho*I site. The reverse primer was 5′-AACCTTGGAAGGATCCTAACTCTATTTACTAAG, containing the underlined *Bam*HI site. This PCR product was digested with *Bam*HI and then ligated to the VSV G tail PCR product. The resulting ligated product was digested with *Xho*I and *Nhe*I and ligated into plasmid pVSV-XN1 to form pVSV-89.6gp160G. The 89.6gp160G gene encodes a hybrid protein with 4 amino acid residues from the cytoplasmic tail of 89.6 gp160 (N-R-V-R) fused to 26 amino acids of the VSV G cytoplasmic tail, beginning with amino acids I-H-L-C.

Recovery of VSV recombinants. Transfections and recoveries were performed as described previously (26). Briefly, baby hamster kidney (BHK) cells were infected at a multiplicity of infection (MOI) of 10 with a recombinant vaccinia virus, vTF7-3 (10), which encodes T7 RNA polymerase. After 1 h, cells were transfected with 3 μg of pBS-N, 5 μg of pBS-P, 1 μg of pBS-L, and 10 μg of either pVSV-XN1, pVSV-gp160, pVSV-gp160G, pVSV-89.6gp160, or pVSV-89.6gp160G by using a cationic liposome reagent (23). After 48 h at 37°C, supernatants from transfected cells were removed, filtered through a 0.2-μm-pore-size filter to remove the majority of vaccinia virus, and transferred to fresh BHK cells. After another 48-h incubation, 1 ml of supernatant was added to fresh BHK cells plated on sterile coverslips; these cells were incubated for 4 to 16 h, and indirect immunofluorescence was performed to assay cells for the expression of VSV G protein or HIV envelope protein.

Indirect immunofluorescence microscopy. The procedures used were essentially those described previously (26). Infected cells on coverslips were fixed in 3% paraformaldehyde. Cells were washed with phosphate-buffered saline (PBS) containing 10 mM glycine and incubated with monoclonal antibody (MAb) I1 binding VSV G (14) and polyclonal sheep anti-gp120 obtained from the AIDS Research and Reference Reagent Program. The secondary antibodies were rhodamine-conjugated goat anti-mouse and fluorescein isothiocyanate-conjugated donkey anti-sheep antibodies (Jackson Immunoresearch). Immunofluorescence microscopy was performed with a Nikon Microphot-FX microscope equipped with a 40× planapochromat objective.

Metabolic labeling of infected cells and virus. BHK cells (2×10^5 to 5×10^5 per plate) on 35-mm-diameter plates were infected with wild-type VSV or recombinants at an MOI of 10 to 100 in 250 μl of Dulbecco's modified Eagle's medium (DMEM) for 30 min. DMEM (2 ml) supplemented with 5% fetal bovine serum (FBS) was added to each plate of cells for 3.5 h at 37°C. The medium was removed, cells were washed twice with PBS, and then methionine-free DMEM containing 100 μCi of [^{35}S]methionine was added to each plate. To prepare labeled cell extracts, radioisotope was added in 0.5 ml of medium for 1 h at 37°C. Cells were washed in PBS and lysed in 0.5 ml of detergent solution (1% Nonidet P-40 [NP-40], 0.4% deoxycholate, 50 mM Tris-HCl [pH 8], 62.5 mM EDTA) with 5 U of aprotinin. To prepare labeled virus, 100 μCi of [^{35}S]methionine was added to infected cells at 4 h after the start of infection in 1.5 ml of methionine-free DMEM supplemented with 1% FBS. Labeling was continued for 16 to 18 h at 37°C. Culture medium was transferred to sterile 1.5-ml Eppendorf tubes and clarified by 10,000-rpm centrifugation in an Eppendorf microcentrifuge for 2 min. Supernatants were layered onto 10% sucrose in TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) and then centrifuged at 180,000 × *g* for 45 min at 4°C in a Beckman SW41 rotor. Virus pellets were resuspended in 300 μl of TE buffer and pelleted through 10% sucrose in a Beckman SW50.1 rotor at 135,000 × *g* for 45 min. Viral pellets were resuspended in 60 μl of TE buffer. Virus and cell lysate samples (10 μl) were analyzed by electrophoresis on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel (12).

For pulse-chase labeling, BHK cells (5×10^5) were plated on 35-mm-diameter dishes and incubated overnight at 37°C. Cells were infected for 3 h at 37°C and then pulse-labeled for 30 min in 0.5 ml of methionine-free medium containing 100 μCi of [^{35}S]methionine. A chase was performed by removing the radioisotope and adding prewarmed DMEM supplemented with 2 mM methionine. Cells and medium were collected immediately after labeling or after a 1- or 4-h chase period. Cells were washed twice in PBS and lysed on the dish in 0.5 ml of detergent solution containing 5 U of aprotinin. Lysates were transferred to 1.5-ml Eppendorf tubes and centrifuged at 10,000 rpm for 2 min to remove nuclei, supernatants were transferred to new tubes, and SDS was added to 0.2%. Culture medium was transferred to 1.5-ml Eppendorf tubes and clarified by centrifugation at 10,000 rpm for 2 min. Supernatants were transferred to new tubes, and detergent solution (1% NP-40, 0.4% deoxycholate, 50 mM Tris-HCl [pH 8]) was added to disrupt virions.

Immunoprecipitations. Polyclonal rabbit anti-VSV antibody (1 to 3 μl) was added to labeled cell lysates and supernatants and incubated at 37°C for 30 min. Protein A-agarose (Gibco BRL) was added, samples were centrifuged at 10,000 rpm in an Eppendorf microcentrifuge for 2 min, and supernatants were transferred to new tubes. Sheep anti-gp120 (1 μl) was added, and samples were incubated at 37°C for 30 to 40 min. Protein A-agarose (40 to 50 μl) was added, and samples were incubated at 37°C for 30 min. Then samples were pelleted, washed three times with ice-cold radioimmunoprecipitation assay buffer (0.1% SDS, 1% deoxycholate, 1% NP-40, and 0.15 M NaCl in 10 mM Tris [pH 7.4]), and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by exposure to a PhosphorImager screen (Molecular Dynamics).

Neutralization assays. HeLa and HeLa-CD4 cells were plated on coverslips at 5×10^5 cells per 35-mm-diameter dish. All antisera used were incubated at 56°C for 1 h to inactivate complement. Viruses (10^7 to 10^8 PFU) were neutralized for 1 h at 37°C with polyclonal rabbit anti-VSV serum to a final dilution of 1:20 (5 μl of antiserum per 100 μl of virus). For neutralizations of both VSV G and HIV gp120, a human polyclonal anti-HIV antibody (HIVIG; AIDS Research and Reference Reagent Program) was added to the virus in addition to the anti-VSV serum at a 1:10 dilution (10 μl per 100 μl of virus). Neutralized virus samples were added to cells in 0.5 ml of DMEM for 1 to 1.5 h at 37°C. DMEM with 5% FBS was added to a total volume of 2 ml, and cells were incubated at 37°C until 8 h after the start of infection. Cells were fixed in 3% paraformaldehyde and examined by indirect immunofluorescence for the expression of VSV G protein.

Electron microscopy and labeling with gold-conjugated antibodies. The procedure used was that described previously (25). Briefly, virus was purified and concentrated by centrifugation twice through 10% sucrose, absorbed onto carbon-coated grids, and blocked with 1% bovine serum albumin in PBS. Virus on grids was stained with anti-VSV G MAb I1 or anti-HIV Env MAbs D47 and D61 (8) and then washed. Grids were stained with goat anti-mouse immunoglobulin G bound to 15-nm gold particles (AuroProbe; Amersham) and washed again. Grids were stained with 2% phosphotungstic acid. Images of viruses on grids were obtained with a Zeiss EM910 electron microscope.

One-step growth curve. BHK cells were plated on 60-mm-diameter plates and infected with viruses (MOI = 40) in 1 ml of DMEM for 30 min at 37°C. Cells were washed five times with 5 ml (each) of PBS to remove all virus. DMEM (3.5 ml) with 5% FBS was then added to cells. Medium (350 μl) was collected at each time point, and 350 μl of DMEM with 5% FBS was added back to each plate to keep the volume constant. Virus titers were determined by plaque assays on BHK cells. Briefly, serial dilutions of each virus were prepared and used to infect BHK cells on six-well plates for 30 min at 37°C. Virus was removed, and cells were overlaid with 0.9% agar and DMEM containing 5% FBS. After 48 h at 37°C, plaques were counted.

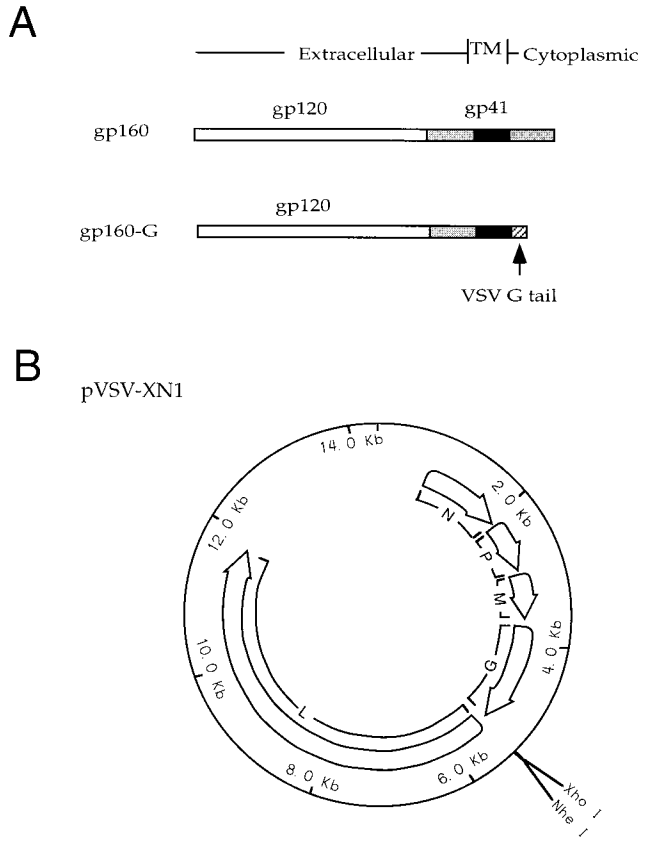

FIG. 1. Diagrams of the proteins expressed in VSV recombinants and the vector used to derive recombinants. (A) Diagram of the gp160 and chimeric gp160G proteins. The positions of the gp120 and gp41 subunits are labeled, and the extracellular, transmembrane (TM), and cytoplasmic domains are indicated. (B) Diagram of the pVSV-XN1 vector, showing the relative positions of the five VSV genes as well as the positions of the *Xho*I and *Nhe*I restriction sites into which the constructs in panel A were cloned. These sites are flanked by VSV transcription start and stop signals.

RESULTS

To generate VSV recombinants expressing the HIV envelope protein (gp160) or a hybrid HIV envelope with the VSV G cytoplasmic domain substituted for the HIV cytoplasmic domain (gp160G), we used a vector described previously that contains the appropriate VSV promoter and terminator sequences flanking unique *Xho*I and *Nhe*I sites (26). The genes to be expressed were generated by PCR and cloned into the vector. The proteins encoded by these genes are diagrammed in Fig. 1A, and a diagram of the expression vector used to derive recombinant VSVs is shown in Fig. 1B. The HIV envelopes for these constructs were derived from the laboratory-adapted LAI strain of HIV (1, 21). Recombinant VSVs expressing either gp160 or gp160G from the new transcription unit were recovered in cells expressing the full-length antigenomic VSV RNA containing the additional gene as well as the VSV N, P, and L proteins (26).

One-step growth curve. The VSV-gp160 and VSV-gp160G viruses were observed to grow to titers lower than that of wild-type VSV and did not cause the typical cytopathic effect, rounding up of cells, as was observed with wild-type VSV (hereafter referred to as VSV). Thus, a one-step growth curve (Fig. 2) was performed to examine the differences in virus production per cell for the two recombinant viruses compared to VSV. Cells were infected simultaneously with each virus at

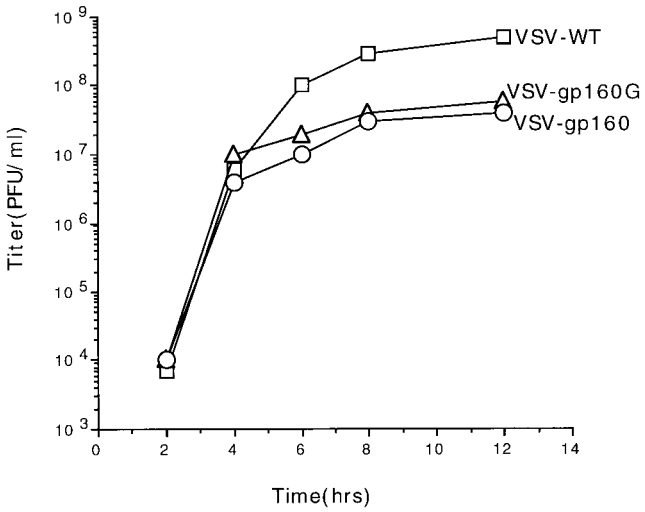

FIG. 2. One-step growth curves of viruses. BHK cells were infected synchronously with wild-type VSV (VSV-WT), VSV-gp160, and VSV-gp160G, and one-step growth curves were determined as described in Materials and Methods. Virus titers in the medium were determined at the times indicated.

an MOI of 40 to allow synchronous infection of all cells. The inoculum was removed, and viral titers were determined subsequently on BHK cells. Both VSV-gp160 and VSV-gp160G replicated at rates similar to that of VSV, but the final titers of both recombinants were about 10-fold lower than that of the wild type.

Expression of gp160 and gp160G from VSV recombinants. To determine whether the recovered VSVs expressed gp160 and gp160G proteins and the proteins were transported to the cell surface, BHK cells were infected with VSV, VSV-gp160, and VSV-gp160G and examined by double-label indirect immunofluorescence for the presence of both VSV G and gp160 or gp160G on the cell surface (Fig. 3). Figure 3A and B show control cells infected with VSV. Infected cells showed surface expression of the VSV G protein (Fig. 3A) and were negative for gp120 expression (Fig. 3B). Figure 3C and D show that cells infected with VSV-gp160 expressed both VSV G and gp120 on their surfaces. The same result was obtained for the VSV-gp160G virus (Fig. 3E and F). These results suggested that the gp160 and gp160G proteins were properly folded because proteins that do not fold correctly are retained inside the cell (6). The transport of gp160 and gp160G to the cell surface appeared to be slower than that of VSV G, as G was readily detected at the cell surface before 4 h postinfection, while gp160 and gp160G were not detected at the cell surface earlier than about 8 h postinfection.

To analyze HIV protein expression, BHK cells were infected with recombinant viruses and labeled for 1 h with [^{35}S]methionine. The HIV envelope protein is synthesized as a precursor gp160 molecule that is subsequently cleaved during transport to gp120 and gp41 (31). Because VSV shuts off host cell protein synthesis, it is possible to analyze labeled cell lysates directly by SDS-PAGE without immunoprecipitation (Fig. 4, lanes 1 through 3). Cells infected with VSV showed five VSV proteins, L, G, N, P, and M (Fig. 4, lane 1), while those infected with VSVgp160 (lane 2) or VSVgp160G (lane 3) showed additional bands of the sizes expected for the gp160 and gp160G proteins. We also performed immunoprecipitations of cell lysates with sheep anti-gp120 serum (Fig. 4, lanes 4 through 6), and these showed that the gp160 (lane 5) and

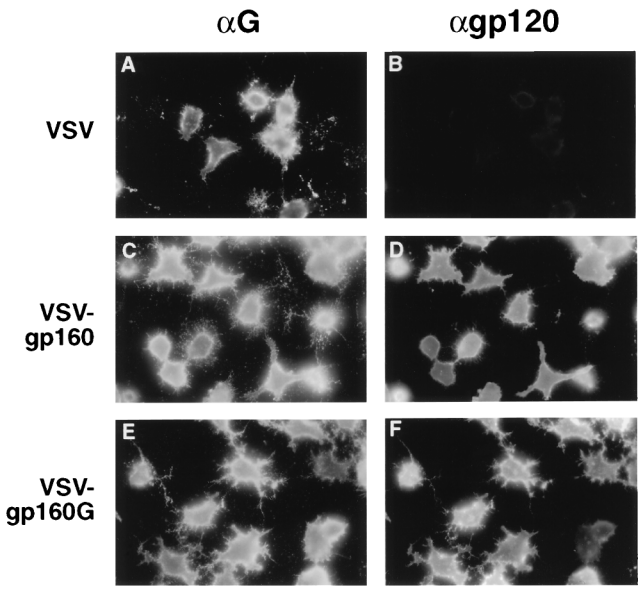

FIG. 3. Surface expression of VSV G and HIV gp120. BHK cells were infected with VSV (A and B), VSV-gp160 (C and D), and VSV-gp160G (E and F). After infection for 16 h, cells were fixed and stained with an anti-VSV G MAb (αG) and sheep polyclonal anti-HIV gp120 serum (αgp120). Secondary antibodies were rhodamine-conjugated donkey anti-mouse (A, C, and E) and fluorescein isothiocyanate-conjugated donkey anti-sheep (B, D, and F) antibodies. Each pair of panels (A and B, C and D, and E and F) presents the same field of cells.

gp160G (lane 6) proteins were significantly enriched after immunoprecipitation, although there was still a high background of VSV proteins in all lanes.

Processing of gp160 and gp160G. To examine the kinetics of gp160 and gp160G processing, we carried out a pulse-chase experiment (Fig. 5). BHK cells were infected with recombinant viruses and labeled with [^{35}S]methionine for 30 min at 3 h after infection. Immunoprecipitations were performed from both cell lysates and culture medium immediately after the pulse or after 1- or 4-h chase periods (Fig. 5). Because the noncovalent interaction between mature subunits gp41 and gp120 is weak, some gp120 is shed into the culture medium after expression of the mature protein at the cell surface. Figure 5 (left panel) shows an infection with wild-type VSV where levels of cell-associated VSV proteins decreased while levels in the supernatant increased over time due to VSV budding. Figure 5 (middle panel) shows the results of the same experiment with the VSV-gp160 virus. The pattern for VSV proteins was the same as that for the wild type, except that HIV gp160 was readily detected in cells and decreased over time as the level of cleaved gp120 subunit increased. The apparent size of the gp160 precursor also decreased slightly over time, probably due to trimming of N-linked oligosaccharides. The level of gp120 released into the medium increased during the 4-h chase period. The same results were obtained with the VSV-gp160G virus (Fig. 5, right panel). Note that the gp160G protein migrated slightly faster than did the full-length gp160 protein because of the shorter cytoplasmic tail in the chimeric protein. We conclude from these studies that the gp160 and gp160G molecules expressed from VSV recombinants are cleaved to their mature forms and transported to the cell surface.

Quantitation of HIV Env protein incorporation into VSV particles. We next determined whether gp160 and gp160G proteins were incorporated into VSV particles. BHK cells were infected with VSV and recombinants and labeled with [^{35}S]methionine. Cell lysates and purified virus were analyzed by SDS-PAGE (Fig. 6). Lysates from cells infected with VSV showed five VSV proteins, as indicated (Fig. 6, lane 1). In cells infected with VSV-gp160, an additional band of the size expected for gp160 appeared below the VSV L protein (Fig. 6, lane 2). Quantitation showed that the amount of gp160 synthesized was 28% of the amount of VSV G protein when corrected for the methionine content of each protein. In cells infected with VSV-gp160G, the labeled gp160G band migrated slightly faster than did the gp160 band because of the shorter cytoplasmic tail (Fig. 6, lane 3). Quantitation of the gp160G protein indicated that it was synthesized at essentially the same level as was the gp160 protein, 31% of the VSV G protein level.

In Fig. 6 (right panel), labeled VSV (lane 1), VSV-gp160 (lane 2), and VSV-gp160G (lane 3) virons are shown. Because gp160 and gp160G are cleaved into their mature subunits before transport to the cell surface, we would expect to find the gp120 and gp41 subunits in virions if they were incorporated. Although there was no detectable gp120 in the VSV-gp160 sample, a low level of gp120 was seen consistently in the VSV-gp160G sample and the level of incorporation was 3% of the level of G protein in virions. We did not detect the hybrid gp41 subunit with the VSV G tail, but this protein would be expected to migrate with the VSV M protein.

Electron microscopy of VSV particles containing HIV Env protein. Previous results indicated that foreign glycoproteins incorporated into recombinant VSVs are distributed over virion surfaces among the VSV G trimers and that recombinants containing extra genes contain longer nucleocapsids (25). We performed electron microscopy with VSV-gp160G to verify the presence of HIV Env protein in VSV particles and to examine the distribution of gp160G in virions. Purified virions

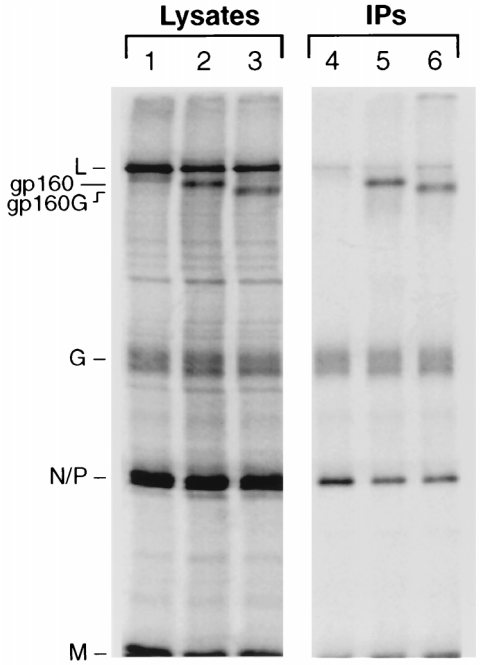

FIG. 4. Expression of gp160 and gp160G in infected cells. BHK cells were infected for 4 h with VSV (lanes 1 and 4), VSV-gp160 (lanes 2 and 5), and VSV-gp160G (lanes 3 and 6) and radiolabeled with [^{35}S]methionine for 1 h. Lysates were analyzed by SDS-PAGE either directly (lanes 1 through 3) or after immunoprecipitation (IPs) with polyclonal sheep anti-gp120 serum (lanes 4 through 6). The positions of the five VSV proteins, as well as gp160 and gp160G, are indicated.

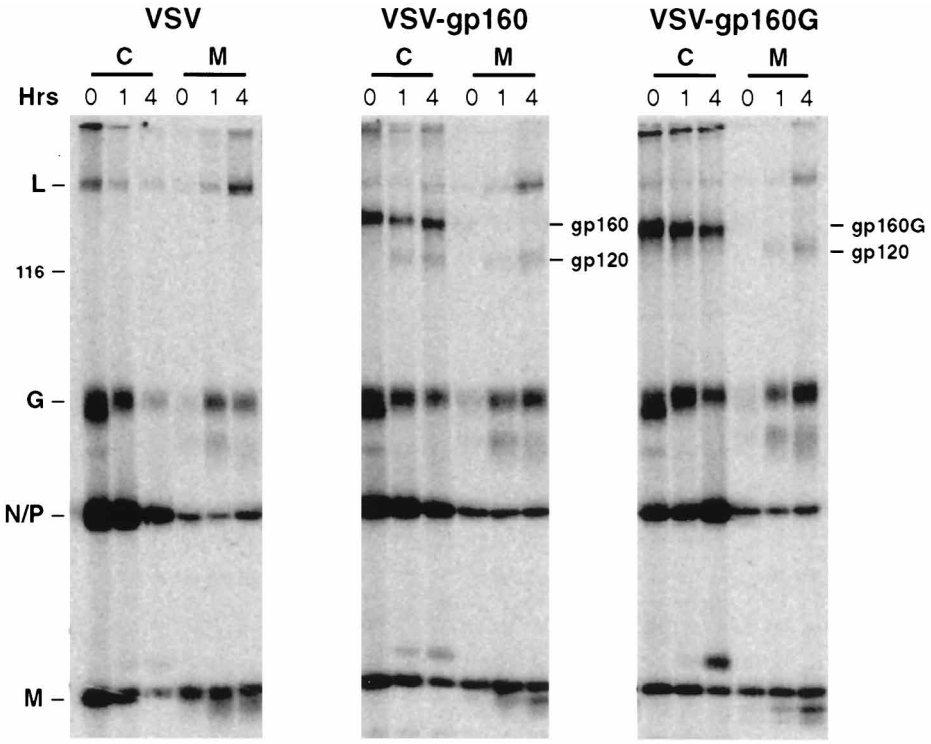

FIG. 5. Immunoprecipitation of gp120 from infected cells and supernatants. BHK cells were infected with VSV (left panel), VSV-gp160 (middle panel), and VSV-gp160G (right panel) for 4 h, labeled with [^{35}S]methionine for 30 min, and chased for 0, 1, or 4 h as indicated. Cell lysates (C) and culture medium (M) were analyzed by SDS-PAGE after immunoprecipitation with polyclonal sheep anti-gp120 serum. The positions of the five VSV proteins, as well as those of gp160, gp160G, gp120, and a 116-kDa marker protein, are indicated. VSV proteins are still visible in immunoprecipitates because of nonspecific binding during immunoprecipitations.

on carbon-coated grids were stained with MAbs directed against VSV G or HIV Env, followed by secondary gold-conjugated antibodies. Viruses were then negative stained with phosphotungstic acid (Fig. 7). All VSV particles were labeled with the anti-G antibody (Fig. 7A) but not with the anti-Env antibodies (Fig. 7B). All VSV-gp160G recombinants were labeled with anti-G antibody (Fig. 7C), and anti-Env antibodies (Fig. 7D) readily detected HIV Env in the majority of particles. The much stronger labeling of VSV G compared to that of HIV Env is consistent with the relatively low incorporation of HIV Env protein compared to that of VSV G (Fig. 6). In addition, the HIV Env protein appeared to be scattered randomly on virions among the VSV G trimers rather than inserted at specific sites. We also noted that the recombinants were longer than was wild-type VSV, reflecting the longer nucleocapsids (containing an additional 2.3 kb in an RNA genome that was originally 11.2 kb).

Specific targeting to CD4$^+$ cells. We next determined whether the gp120 protein in VSV-gp160G virions could function to allow entry via the HIV receptor CD4 (15). We used HeLa cells expressing CD4 for these experiments because HeLa cells are also known to express the fusin-CXCR4 cofactor required for HIV entry (9). Because VSV G protein is also present in virions and allows entry into all cells, it was first necessary to neutralize infectivity due to VSV G protein with anti-VSV serum.

We initially titrated our anti-VSV serum to find an amount that was sufficient to completely neutralize infectivity on HeLa cells. HeLa cells and HeLa-CD4 cells were then infected either with neutralized VSV and VSV-gp160G viruses or with nonneutralized controls. After 8 h, infected cells were examined for infection by indirect immunofluorescence microscopy for

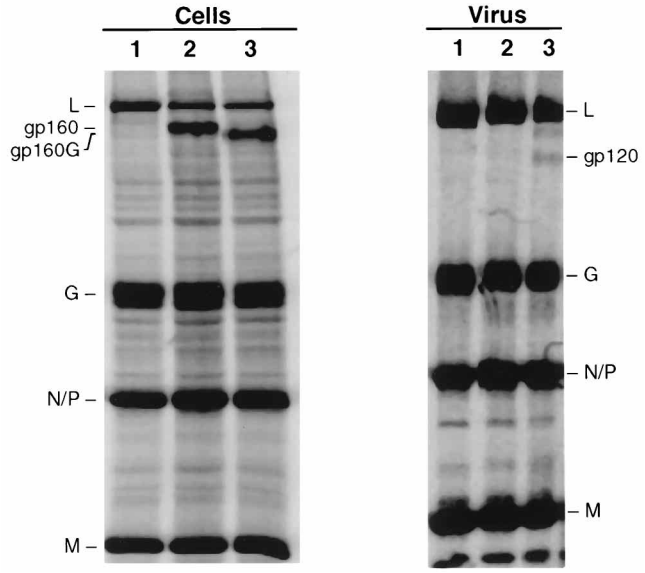

FIG. 6. Expression of gp160 and gp160G in cells and incorporation into virions. BHK cells were infected for 4 h with VSV (lanes 1), VSV-gp160 (lanes 2), and VSV-gp160G (lanes 3), labeled with [^{35}S]methionine for 1 h, and lysed. To label virus, infected cells were labeled overnight and supernatants were subjected to centrifugation twice through 10% sucrose. Cell lysates (left panel) and labeled virus (right panel) were then analyzed by SDS-PAGE. The relative positions of VSV proteins, as well as gp160, gp160G, and gp120, are indicated.

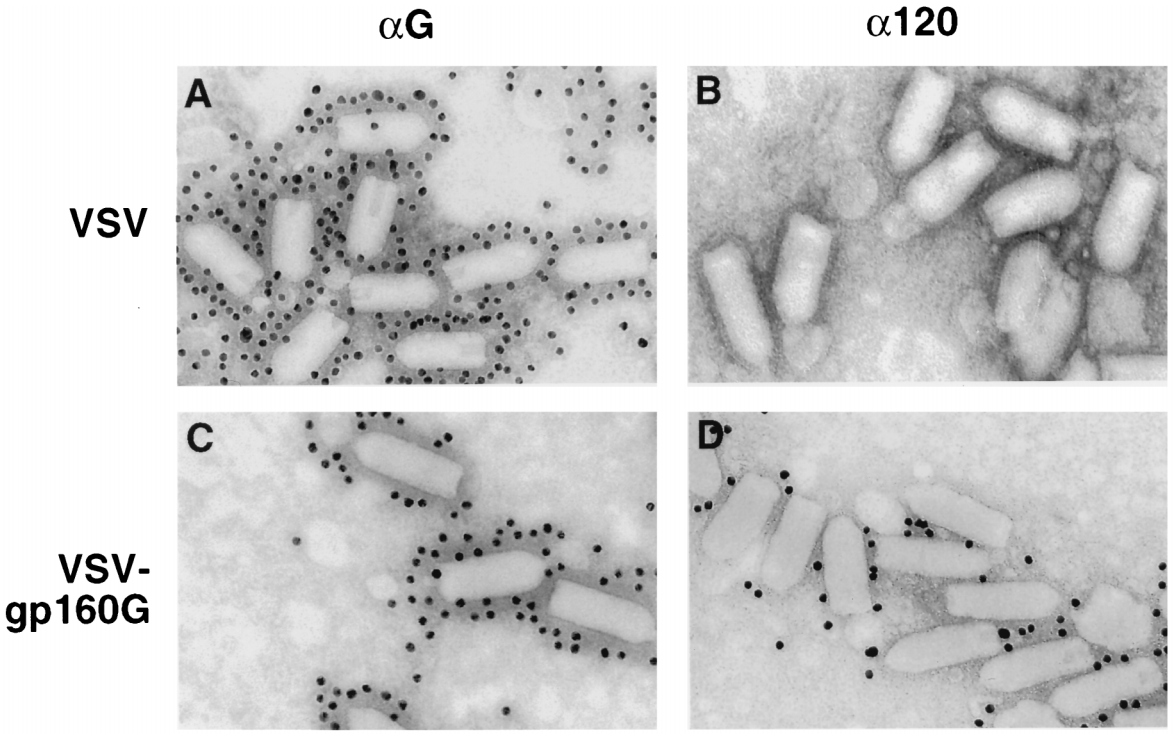

FIG. 7. Electron microscopy of VSV labeled with gold-conjugated antibodies. Purified VSV (A and B) and VSV-gp160G (C and D) virions were adsorbed to carbon-coated grids, labeled with an anti-G MAb (αG; A and C) or anti-Env MAbs (α120; B and D), labeled with anti-mouse immunoglobulin G conjugated to gold particles, and negative stained.

the expression of VSV G protein (Fig. 8). Without neutralization, both wild-type VSV and VSV-gp160G infected HeLa and HeLa-CD4 cells (Fig. 8, panels 1, 2, 9, and 10). In the presence of excess anti-VSV serum, wild-type VSV did not infect either HeLa or HeLa-CD4 cells (Fig. 8, panels 3 and 4), while VSV-gp160G was completely neutralized on HeLa cells (panel 11) but still showed infectivity on HeLa-CD4 cells (panel 12). To determine whether the infectivity on HeLa-CD4 cells was due to entry via gp120, we added both anti-VSV serum and anti-HIV serum. This combination completely neutralized the infectivity of VSV-gp160G on HeLa-CD4 cells (Fig. 8, panel 16). The infectivity of VSV on either cell type was of course completely neutralized by anti-VSV serum + anti-HIV serum (Fig. 8, panels 7 and 8), as was the infectivity of VSV-gp160G on HeLa cells (panel 15). To rule out nonspecific effects of the anti-HIV serum, we verified that it had no effect on the infectivity of VSV or VSV-gp160G on HeLa or HeLa-CD4 cells in the absence of anti-VSV serum (Fig. 8, panels 5, 6, 13, and 14). Because the VSVgp160G virus can enter via the VSV receptor, the addition of anti-HIV serum would not be expected to neutralize this virus in the absence of anti-VSV serum. These results demonstrated specific entry of the VSV-gp160G recombinant via the CD4 receptor.

A time course of infection monitored by immunofluorescence with a polyclonal anti-VSV antibody revealed that the initiation of infection by the VSVgp160G recombinant via the gp120-CD4 pathway was much slower than that observed via VSV G. The reason for the slower infection is not known, but it may indicate slower fusion by the HIV envelope protein or slower activation of VSV cores that enter cells via cell surface fusion rather than the normal endosomal route.

In order to estimate the specific titers of recombinants entering via the CD4 receptor, cells were infected with various amounts of VSV-gp160G virus (preneutralized with anti-VSV serum) and stained in an immunofluorescence assay, and the number of cells infected before virus spread was counted. This method gave titers in the range of 1×10^5 to 3×10^5 infectious particles per ml for VSV-gp160G. The virus titer on HeLa cells without preneutralization of G infectivity was 3×10^7 infectious particles per ml.

Although we had not been able to observe gp120 in virions derived from the VSV-gp160 virus, we performed a similar experiment with these virions to determine whether there was any specific infection of HeLa-CD4 cells after neutralization with anti-VSV serum. We did in fact observe a very low level of HeLa-CD4-specific infectivity with the VSV gp160 virus, corresponding to a titer of approximately 10^3 infectious particles per ml. This result indicates that a small amount of gp160 must have been incorporated into VSV particles and that by biochemical methods, it was simply not detectable above the background.

Recombinant VSV expressing the envelope protein of an HIV-1 primary isolate. HIV-1 isolates that have been adapted to growth in transformed T-cell lines and extensively passaged are commonly referred to as laboratory-adapted HIV-1 isolates. Such isolates differ significantly from primary HIV-1 isolates, which have been minimally passaged only in peripheral blood mononuclear cells. Many primary isolates cannot be propagated initially in transformed T-cell lines (2) and must be passaged extensively before they become adapted. This adaptation has been correlated with mutations of the viral envelope protein, changes in epitope exposure, and alterations of viral phenotype (17, 20, 29, 32). Typically, primary isolates are relatively insensitive to neutralization by antisera produced against the envelope proteins of HIV laboratory isolates (18, 33). Because we are interested in vaccine applications for

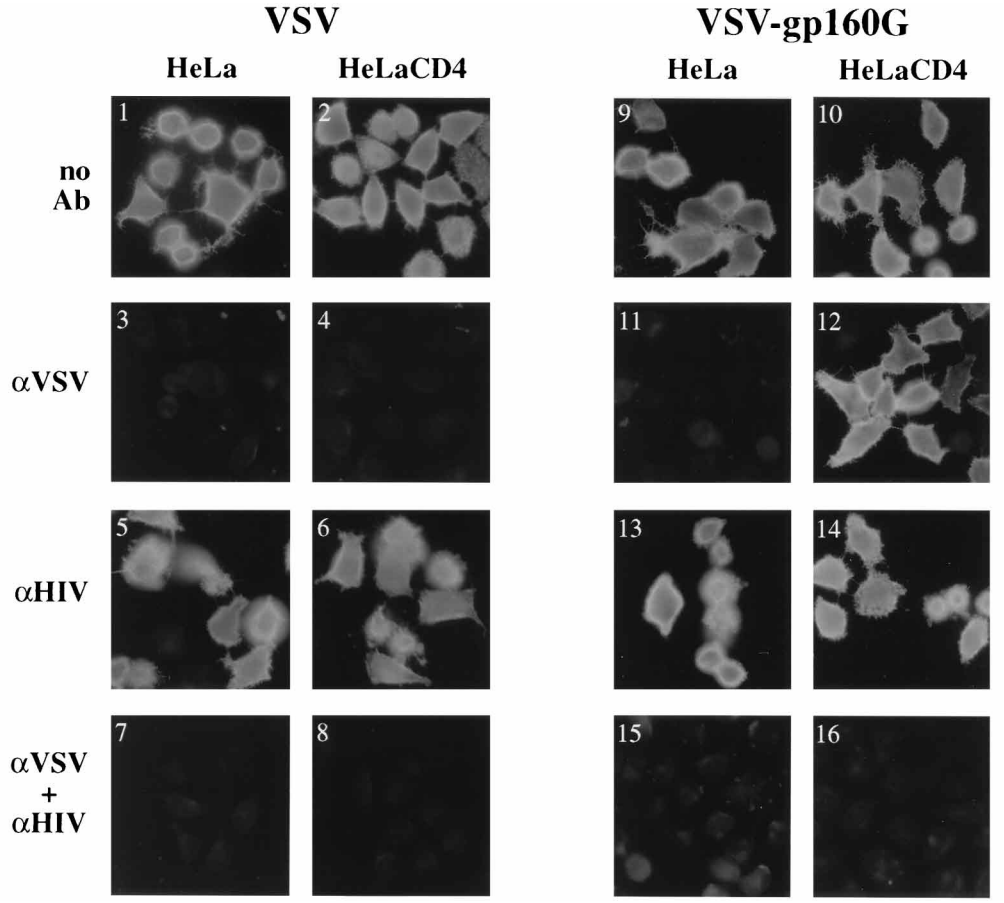

FIG. 8. Specific infection of $CD4^+$ cells after neutralization of VSV G. VSV (panels 1 through 8) and VSV-gp160G (panels 9 through 16) were incubated without antibody (no Ab; panels 1, 2, 9, and 10) or with rabbit polyclonal anti-VSV antibody alone (αVSV; panels 3, 4, 11, and 12), human polyclonal anti-HIV antibody alone (αHIV; panels 5, 6, 13, and 14), or both (αVSV + αHIV; panels 7, 8, 15, and 16) as indicated. Neutralized virus was then added to HeLa (panels 1, 3, 5, 7, 9, 11, 13, and 15) and HeLa-CD4 (panels 2, 4, 6, 8, 10, 12, 14, and 16) cells, which were fixed after 8 h and stained by immunofluorescence with a mixture of anti-VSV G MAbs and rhodamine-conjugated donkey anti-mouse antibodies.

VSV-HIV recombinants and all of our initial experiments were done with the envelope protein of a laboratory strain, we decided to generate the equivalent constructs from a primary isolate, 89.6, for subsequent analysis in animals. An infectious clone has been generated from the 89.6 isolate, a virus that is dual tropic and highly cytopathic in monocytes and peripheral blood mononuclear cells (4). The dual tropism of this virus has been shown to be due to its ability to use both the fusin and CKR coreceptors (7).

Recombinant VSVs expressing the 89.6 gp160 or 89.6 gp160G hybrid proteins were recovered and used to infect BHK cells. Infected cells were labeled with [^{35}S]methionine, and labeled cells and virus were collected and analyzed by SDS-PAGE (Fig. 9). Cells infected with VSV (Fig. 9, lane 1) showed the indicated bands of VSV proteins, while those infected with the recombinants VSV-89.6gp160 (lane 2) and VSV-89.6gp160G (lane 3) showed the expected extra bands of HIV envelope proteins. The level of 89.6 gp160 expression was 25% of that of VSV G, while 89.6 gp160G expression was 17% of that of G. To determine whether the 89.6 envelope also required the G cytoplasmic tail for incorporation into VSV particles, we examined purified virus particles (Fig. 9, right panel). Just as for the LAI envelope, gp120 was detected only in virions produced by VSV-89.6gp160G (Fig. 9, lane 3), not in those produced by VSV-89.6gp160 (lane 2). The level of gp120 incorporation was 3.2% of that of VSV G. We found also that VSV-89.6gp160G was able to specifically infect HeLa-CD4 cells after neutralizing infectivity due to VSV G.

DISCUSSION

The results presented here demonstrate that replication-competent recombinant VSVs containing large foreign genes can be generated. The recombinants described here expressed HIV-1 envelope glycoprotein genes of 2.5 kb or slightly smaller chimeric genes encoding the HIV glycoprotein with its cytoplasmic domain replaced with the cytoplasmic domain of the VSV G protein. Although both foreign proteins were expressed at high levels, proteolytically cleaved, and transported to the cell surface, only the hybrid protein with the VSV G tail was incorporated into virions at readily detectable levels. The envelope protein was clearly functional because these VSV-gp160G viruses specifically infected cells expressing the HIV receptor CD4 when the infectivity due to the VSV envelope protein was neutralized.

Recent studies from our laboratory have shown that several different cellular or viral membrane glycoproteins unrelated to VSV G protein are incorporated into VSV particles at levels of up to 30% of that of VSV G itself when expressed from the VSV genome, yet none of these proteins required the VSV G

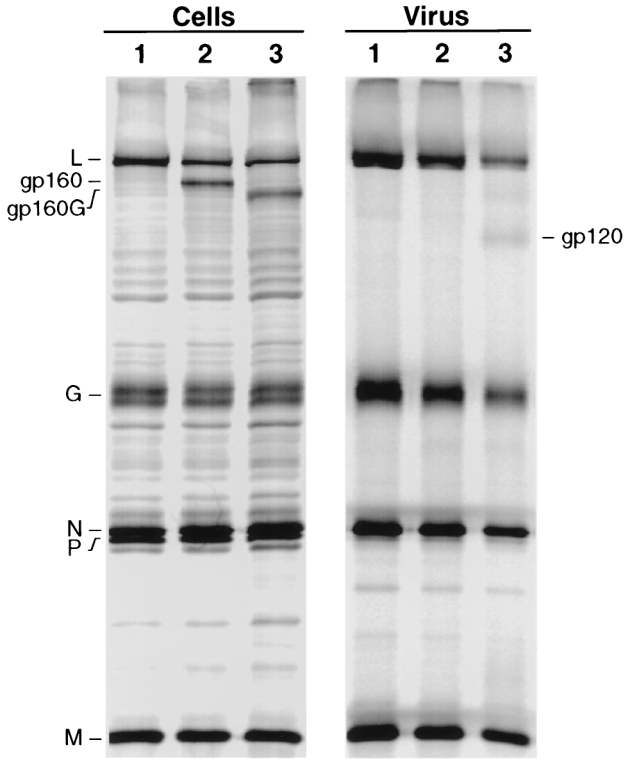

FIG. 9. Expression of 89.6 gp160 and 89.6 gp160G. BHK cells were infected with VSV (lanes 1), VSV-89.6gp160 (lanes 2), and VSV-89.6gp160G (lanes 3) for 4 h. Cells were labeled with [^{35}S]methionine for 1 h. To prepare labeled virus, infected cells were labeled overnight and supernatants were subjected twice to centrifugation through 10% sucrose. Cell lysates (left panel) and labeled virus (right panel) were then analyzed by SDS-PAGE. The relative positions of VSV proteins, as well as 89.6 gp160, 89.6 gp160G, and 89.6 gp120, are indicated.

tail for incorporation. Why then is the VSV G tail required for incorporation of the HIV-1 envelope protein? One simple model is that the normal 150-amino-acid cytoplasmic domain of the HIV envelope is too large to be accommodated between the viral membrane and internal matrix or nucleocapsid proteins of VSV. However, earlier complementation studies argue against this model because an HIV-1 Env protein with a truncated cytoplasmic tail the size of the VSV G tail (29 amino acids) was not incorporated into VSV particles lacking VSV G (21). A second model is that the HIV-1 Env protein contains a signal which directs it to localize in regions of the plasma membrane distinct from the sites of VSV budding. The replacement with the VSV tail could provide a stronger positive signal to redirect interaction of the HIV Env-G protein with VSV matrix or nucleocapsid proteins at the site of VSV budding, or it could eliminate a signal present in the membrane-proximal amino acids of the HIV envelope protein which normally prevent it from reaching sites of VSV budding. This latter model may be testable with additional recombinants.

Although the level of expression of Env-G chimeras within infected cells was quite high (30% of that of VSV G itself), the incorporation into VSV particles was only about 3% of that of VSV G. One possible reason for this relatively low incorporation is the intracellular retention and degradation of up to 90% of the HIV-1 envelope protein before it reaches the cell surface (31). The transport of gp160 to the cell surface is also very slow compared to that of VSV G; therefore, much of the virus budding early after infection may be preferentially enriched in VSV G. In addition, the interaction between mature gp120 and gp41 is weak, and some of the gp120 may have been lost during particle purification. With another VSV recombinant expressing the measles virus F glycoprotein, which is also poorly transported, we also noted poor incorporation into VSV (25). By incorporating mutations that slow the transport of VSV G protein, it may be possible to obtain virions containing higher proportions of slowly transported foreign envelope proteins.

Although we did not detect the incorporation of wild-type Env protein into VSV particles by SDS-PAGE analysis, some must have been incorporated because a very low titer of CD4-specific infectivity ($\sim 10^3$ infectious particles/ml) was detected in these stocks. Earlier studies have shown clearly that growth of VSV and HIV-1 in the same cells does generate VSV (HIV-1) pseudotypes that specifically infect $CD4^+$ cells (5). The pseudotype titers obtained in those studies were in fact comparable to those obtained with the VSV-gp160 recombinant and probably reflect the very poor incorporation of HIV-1 Env protein into VSV virions. The substitution of the VSV tail in the VSV-gp160G recombinant increases the CD4-specific pseudotype titers at least 100-fold apparently by increasing the incorporation of HIV Env into VSV virions. The relatively high titers of pseudotypes (3×10^5/ml) obtained with the VSV-gp160G recombinant could be very useful for analysis of inhibitors of HIV-1 entry.

As we have observed for most other recombinants, the VSV-HIV recombinants were stable for at least six low-multiplicity passages involving a total amplification of 10^{30}, but the viral titers obtained in one-step growth curves were approximately 10-fold-lower than that of wild-type VSV. The reduction in titers of VSV-HIV recombinants could be due to saturation of the cellular glycosylation or folding machinery by the heavily glycosylated Env protein. Indeed, oligosaccharide processing on VSV G protein appeared to be retarded in VSV-HIV recombinants, suggesting effects of high-level Env protein expression on transit through the exocytic pathway. We also noted a greatly decreased cytopathic effect in the recombinants which could be related to reduced virus budding. Titers achieved with other VSV recombinants have depended on what foreign genes were expressed. VSV expressing the bacterial chloramphenicol acetyltransferase gene grew to wild-type titers (26), while titers of VSV expressing the cellular CD4 protein were reduced about 10-fold. VSV expressing the measles virus F protein grew to extremely low titers but rapidly returned to high titers after the loss of F protein expression (25). In contrast, VSV expressing the influenza virus hemagglutinin protein grew to wild-type titers (11a).

One of the possible applications anticipated for these or similar VSV-HIV recombinants is as HIV vaccines. Because VSV infects humans (laboratory strains typically cause only mild or asymptomatic infections), mucosal or other routes of infection could generate strong humoral and cellular immune responses. Because the titers of VSV-HIV recombinants are reduced compared to that of wild-type virus, it is also likely that they will display reduced pathogenicity, but additional attenuating mutations could be introduced if necessary. Alternatively, inactivated VSV particles displaying functional HIV envelope oligomers might also induce virus-neutralizing antibodies. Because VSV itself encodes only five structural proteins to compete for the immune response, these vectors could have significant advantages over much more complex vectors, such as those based on poxviruses, which encode large numbers of proteins that may compete in the immune response (19). Studies to evaluate immune responses to VSV-HIV recombinants in animals are in progress.

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