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Cytoplasmic Domain Requirement for Incorporation of a Foreign Envelope Protein into Vesicular Stomatitis Virus

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Incorporation of human immunodeficiency virus type 1 (HIV-1) envelope proteins into vesicular stomatitis virus (VSV) particles was studied in a system that allows expressed envelope proteins to rescue phenotypically a temperature-sensitive mutant of VSV (*ts*O45). This mutant exhibits defective transport of its own envelope glycoprotein (G) and can be rescued by simultaneous expression of wild-type G protein from cDNA. We report here that a hybrid HIV-1–VSV protein containing the extracellular and transmembrane domains of the HIV-1 envelope protein fused to the cytoplasmic domain of VSV G protein was able to rescue the *ts*O45 mutant lacking the G protein, while the wild-type HIV-1 envelope protein was not. The VSV(HIV) pseudotypes obtained infected only CD4$^+$ cells and were neutralized specifically by anti-HIV-1 sera. Our results indicate that the cytoplasmic tail of the VSV glycoprotein contains an independent signal capable of directing a foreign protein into VSV particles. The VSV(HIV) pseudotypes generated here were prepared in the absence of HIV-1 and should be useful for identifying molecules that block HIV-1 entry.

Assembly of viral glycoproteins into a membrane envelope during virus budding is a fundamental but poorly understood process. For some viruses, assembly is thought to require specific interactions between the cytoplasmic tail of the viral glycoprotein(s) and internal viral components gathered on the cytoplasmic side of the plasma membrane. For example, there is evidence of interaction between the cytoplasmic tail of alphavirus spike glycoproteins and nucleocapsid proteins (10, 29), and deletion of the cytoplasmic tail from the vesicular stomatitis virus (VSV) glycoprotein (G) reduces its incorporation into VSV particles (25). In contrast, the cytoplasmic tails of the Rous sarcoma virus glycoprotein and human immunodeficiency virus type 1 (HIV-1) envelope proteins apparently are not required for assembly into virus particles (5, 13).

VSV, the prototype rhabdovirus, contains a negative-strand RNA genome encoding five structural proteins. These are the membrane-spanning envelope glycoprotein (G), a matrix protein (M), and three genome-associated proteins, N, NS (or P), and L (reviewed in reference 16). We have described previously a system in which a temperature-sensitive G protein mutant of VSV (*ts*O45) (3) can be phenotypically rescued by coexpression of the wild-type VSV glycoprotein from cloned cDNA (25). This study suggested that the VSV G cytoplasmic domain contained an essential recognition site for assembly because VSV G proteins lacking this 29-amino-acid C-terminal domain or containing foreign cytoplasmic domains were not incorporated into VSV particles. We performed the experiments described here to determine whether the VSV G cytoplasmic domain was sufficient to direct a foreign protein, the HIV-1 envelope glycoprotein (Env), into VSV particles.

The HIV-1 Env protein is synthesized as a precursor (gp160) that is cleaved during transport to the plasma membrane into two noncovalently associated subunits, gp120 and gp41 (23, 26). The gp120 subunit is found on the external surface of the virions and contains the binding site for the HIV receptor, CD4. The gp41 subunit contains the membrane-spanning domain and the cytoplasmic domain (6, 9, 22).

MATERIALS AND METHODS

Viruses and cells. All cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Vaccinia virus, strain vTF7-3 (obtained from B. Moss, National Institute of Allergy and Infectious Diseases, Bethesda, Md.), was propagated in HeLa cells. VSV, strain *ts*O45, was propagated in baby hamster kidney (BHK) cells. CD4$^+$ HeLa cells were obtained through the AIDS Research and Reference Reagent Program.

Chimeric gene construction. Construction of the plasmid pEnv-wt containing the BH10 strain of the HIV-1 envelope glycoprotein under control of the bacteriophage T7 promoter has been described previously (12). pEnv-G 709 was made by polymerase chain reaction (PCR) amplification (17) with a primer (5′-TACACAAGCTTAATACACTC-3′) overlapping a *Hin*dIII restriction site (underlined) in the HIV-1 Env ectodomain coding region and a second primer (5′-ATTA TCGGATCCTAACTCTATT-3′) that overlapped the junction between the transmembrane and cytoplasmic tail coding domain and introduced a *Bam*HI site (underlined). A *Bam*HI-*Hin*dIII fragment encoding the 26 C-terminal amino acids of VSV G was purified from pTMB (14). The PCR product was digested with *Hin*dIII and *Bam*HI and ligated to the VSV G fragment along with pEnv-wt that had been digested with *Hin*dIII. The pEnv-G 751 construct was prepared in the same way except that the second primer (5′-TGCTAGGATCCCGTTCACTAA-3′) overlapped the sequence encoding amino acids 748 to 754 in the HIV-1 Env cytoplasmic domain. pEnv-tr 735 was made by using a second primer (5′-CTCTCAAGCTTATTCTTCTATTCC-3′) that introduced a stop codon (bold letters, antisense) to truncate the HIV cytoplasmic domain at amino acid position 735, followed by a *Hin*dIII site (underlined). This PCR product was ligated to pEnv-wt that had been digested with *Hin*dIII. The DNA sequences of all regions generated by

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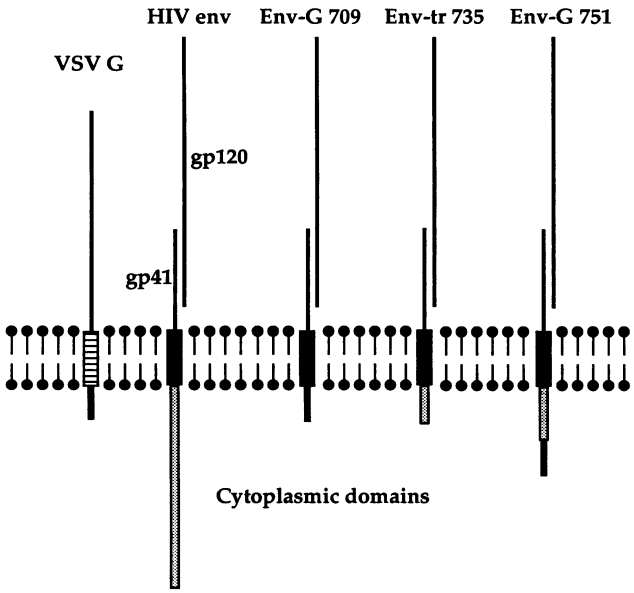

FIG. 1. Structures of wild-type and hybrid envelope proteins. A schematic view of each envelope protein is shown to compare external, transmembrane, and cytoplasmic domains and to illustrate those regions used in hybrid protein construction (■, cytoplasmic domain of VSV G; ▨, cytoplasmic domain of HIV Env). The lipid bilayer ( ) and the membrane-spanning domains (▮, VSV G; ▮, HIV Env) are indicated. A detailed description of the plasmid constructs is given in Materials and Methods.

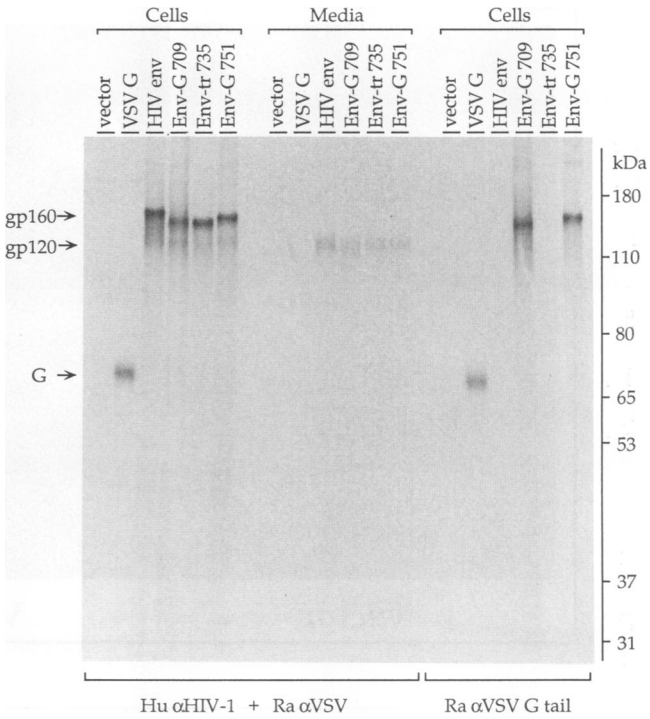

FIG. 2. Expression of envelope proteins. ^{35}S-labeled proteins from media and cell lysates expressing each of the indicated envelope protein constructs were analyzed by immunoprecipitation and SDS-PAGE. A mixture of polyclonal rabbit anti-VSV (Ra αVSV) and human anti-HIV-1 (Hu αHIV) sera was used to immunoprecipitate cell lysates (left six lanes labeled Cells) and medium samples (center six lanes labeled Media), and a polyclonal rabbit antiserum raised against a peptide corresponding to the VSV G cytoplasmic domain was used to immunoprecipitate cell lysates (six lanes on the right labeled Cells). The sizes (in kilodaltons) of the proteins are indicated on the right.

PCR were confirmed by dideoxynucleotide sequencing (18) with Sequenase (U.S. Biochemical Corp.)

Expression and radioimmunoprecipitation of envelope proteins. BHK cells were infected with vTF7-3 at a multiplicity of infection of 5. After a 30-min adsorption period, the cells were transfected with various plasmids with the use of TransfectACE (Life Sciences, Inc.). At 6 h postinfection, samples were pulse-labeled with 100 μCi of [^{35}S]methionine for 30 min and chased for 4 h in the presence of excess unlabeled methionine. Medium samples were collected, and the cells were disrupted in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Nonidet P-40); samples were spun in a microcentrifuge at 10,000 × *g* for 2 min to remove cell debris. Sodium dodecyl sulfate (SDS) was added to a 0.2% final concentration in the cell lysates, and the medium samples were adjusted to 1% Nonidet-40 and 0.2% SDS. Samples were incubated at 4°C for 18 h in the presence of specific antisera, and immune complexes were precipitated with excess fixed *Staphylococcus aureus* (Pansorbin; Calbiochem) and analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE).

Indirect surface immunofluorescence. BHK cells were infected and transfected as described above and incubated at 37°C for 10 h. The cells were fixed in 3% paraformaldehyde and washed with phosphate-buffered saline containing 10 mM glycine (PBS-Gly). The cells were then incubated with primary antibody (diluted 1:100) and then with fluorescein isothiocyanate-conjugated secondary antibody (diluted 1:100) for 20 min at room temperature. Samples were washed three times with PBS-Gly after incubation with each antibody.

Rescue and titration of *ts*O45. BHK cells were infected with *ts*O45 and vTF7-3 at an multiplicity of infection of 5 for each virus and transfected with plasmids encoding the envelope proteins. After incubation at 40.5°C for 18 h, medium samples were collected and cleared of cells and debris by low-speed centrifugation. The titers of the supernatants were determined on confluent monolayers of HeLa and CD4$^+$ HeLa cells. After a 30-min adsorption period, an agar overlay was added and the cells were incubated at 32°C for 1 to 2 days.

RESULTS

In initial experiments, we found that expression of the HIV-1 Env protein was not able to generate VSV(HIV) pseudotypes with the *ts*O45 mutant of VSV at the nonpermissive temperature. Such pseudotypes would be expected to have the VSV core surrounded by an envelope containing the HIV glycoprotein. To test the possibility that the failure to obtain pseudotypes resulted from the lack of a specific incorporation signal on HIV-1 Env, we prepared a construct encoding the HIV-1 Env protein with its normal cytoplasmic domain replaced by the VSV G cytoplasmic domain (Fig. 1). This construct (Env-G 709) has the 26 C-terminal amino acids of the VSV G protein replacing the entire cytoplasmic domain of HIV-1 Env (residues 710 to 856). As a control, we prepared a second construct (Env-tr 735) encoding a truncated HIV-1 Env protein with a cytoplasmic tail the same

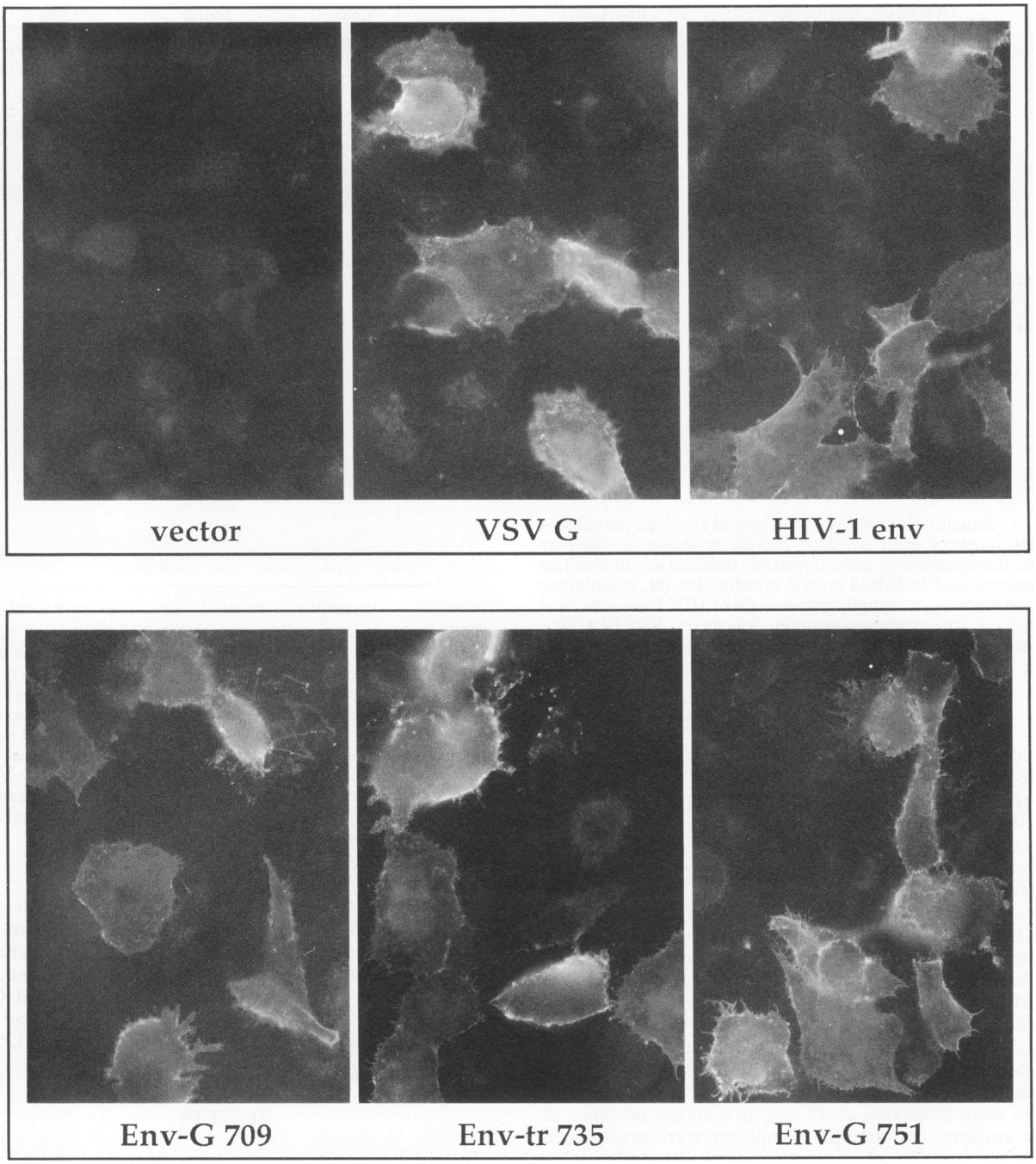

FIG. 3. Indirect surface immunofluorescence. BHK cells were infected with vTF7-3 and transfected with the various envelope constructs. Samples were fixed and stained for surface expression at 10 h posttransfection. A polyclonal sheep anti-gp120 serum was used as a primary antibody, and fluorescein isothiocyanate-conjugated donkey anti-sheep immunoglobulin G was used as a secondary antibody (for HIV-1 Env and vector samples). For the VSV G sample, polyclonal rabbit anti-VSV (primary) and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (secondary) were used.

length as that in Env-G 709. A third construct (Env-G 751) encodes an HIV-1 Env protein, with the 26 amino acids from VSV G replacing residues 752 to 856 in the HIV-1 Env cytoplasmic tail.

Hybrid protein expression. To verify transport and cleavage of the hybrid proteins, these constructs were expressed under control of the bacteriophage T7 promoter in HeLa cells infected with a recombinant vaccinia virus (vTF7-3) that expresses T7 RNA polymerase (4). Immunoprecipitated, [^{35}S]methionine-labeled proteins were analyzed by SDS-PAGE (Fig. 2). Each mutant protein was expressed at a level comparable to that of the HIV-1 Env protein. To ensure that the cytoplasmic tail of G was present on the hybrid constructs, we immunoprecipitated samples by using

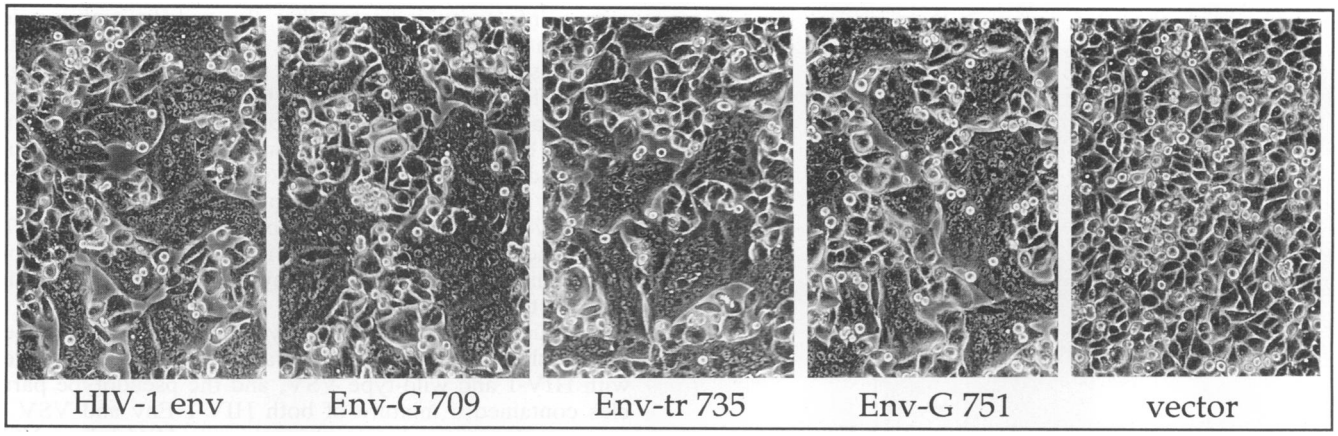

FIG. 4. Syncytium formation in CD4$^+$ HeLa cells. Functional analysis of the HIV-1 Env constructs was performed to determine whether they retained the ability to cause cell fusion. CD4$^+$ HeLa cells were infected with vTF7-3 and transfected with the indicated plasmids. The cells were then incubated at 37°C for 12 h and photographed.

a rabbit antiserum specific for the G cytoplasmic domain (Fig. 2). As expected, VSV G, Env-G 709, and Env-G 751 all precipitated with the antipeptide serum to the VSV G cytoplasmic domain, while the HIV-1 Env and Env-tr 735 did not. The small differences in mobilities of the proteins were consistent with predicted molecular weights. In addition, each of the mutant protein precursors was cleaved to generate gp120, which was immunoprecipitated from the medium (Fig. 2). Because of the loose association between gp41 and gp120, gp120 is readily released into the culture medium (15, 19). Because processing of HIV-1 gp160 occurs late during transport (26), these results suggested that the mutant proteins as well as the HIV-1 Env protein were transported.

We examined expression of the envelope proteins by indirect surface immunofluorescence microscopy and found that the mutant envelope proteins were expressed on the plasma membrane at levels similar to those of wild-type HIV-1 Env (Fig. 3). Equivalent levels of surface expression were also verified by flow cytometry. When expressed in CD4$^+$ HeLa cells (8), each mutant protein caused formation of syncytia equivalent to that produced by the wild-type HIV-1 protein (Fig. 4). Taken together, these results indicate that the various mutations introduced into the HIV-1 envelope protein had no effect on proteolytic processing, levels of surface expression, or fusogenicity.

Phenotypic rescue. We next determined whether any of the mutant proteins could rescue infectivity of VSV *ts*O45 by forming VSV(HIV) pseudotypes. Cells were infected with both vTF7-3 and *ts*O45 and then transfected with plasmids encoding wild-type or hybrid Env proteins or the VSV G protein. The cells were maintained overnight at the nonpermissive temperature (40.5°C) to prevent expression of the *ts*O45 G protein at the plasma membrane. The viral titers in samples of the medium were then determined on HeLa and CD4$^+$ HeLa cells at 32°C, the permissive temperature for *ts*O45 (Table 1). Rescue by the VSV G protein yielded similar titers with both HeLa and CD4$^+$ HeLa cells. These titers are lower than those observed previously for mouse L cells (25), because VSV plaquing efficiency is much lower on HeLa cells and because the experimental protocol was optimized for rescue by the HIV envelope proteins. Of the three HIV-related proteins, only the Env-G 709 chimeric protein (which has the VSV G tail appended directly to the HIV-1 Env transmembrane domain) yielded a titer, and this was specific for CD4$^+$ HeLa cells. To determine whether the infectivity rescued by Env-G 709 resulted from the HIV envelope protein, we analyzed neutralization by anti-HIV sera. Infectivity rescued with VSV G protein was neutralized only by anti-VSV serum, while infectivity rescued with Env-G 709 was neutralized by anti-HIV serum and not by anti-VSV serum (Table 1). This result indicates that infectivity was due to the Env-G 709 protein.

Analysis of protein in virus particles. We also analyzed [^{35}S]methionine-labeled proteins in virus pelleted from medium samples to determine whether HIV-1 Env protein was detectable (Fig. 5). Viral proteins were immunoprecipitated with antiserum to both VSV and HIV and analyzed by

TABLE 1. Rescue of *ts*O45 by HIV-1–VSV hybrid proteins

DNA	Titer (PFU/ml)[a] with:			
	HeLa cells	CD4$^+$ HeLa cells	Anti-VSV serum	Anti-HIV serum
Env wt[b]	0	0		
Env-G 709	0	1.1×10^3–3.0×10^3	2.0×10^3–2.8×10^3	0
Env-tr 735	0	0		
Env-G 751	0	0		
VSV G	1.1×10^3–6.0×10^3	1.5×10^3–5.0×10^3	0	1.0×10^3–5.0×10^3
Vector only	0	0		

[a] The range of titers observed in five experiments is given. For neutralization experiments, supernatants were incubated with 1:100 dilutions of either rabbit anti-VSV serum (Indiana serotype) or a pool of human anti-HIV-1 serum at 37°C for 1 h, and titers on CD4$^+$ HeLa cells were determined at 32°C.
[b] wt, wild type.

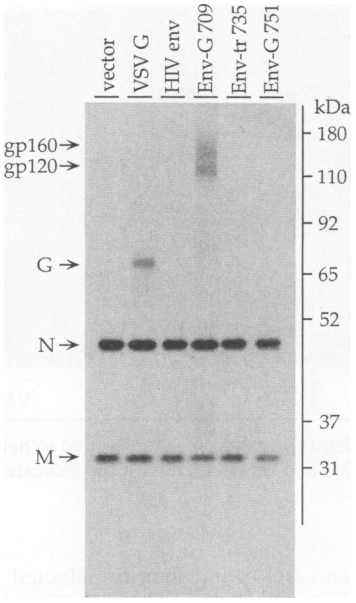

FIG. 5. Analysis of virion-associated proteins. Rescue of *ts*O45 was performed as described in Methods and Materials except that 200 μCi of [^{35}S]labeled methionine was added to the culture medium 4 h postinfection. Virions were purified by centrifugation through 10% sucrose at 49,000 × *g* for 90 min in a Beckman SW41 rotor. The pellet was resuspended in lysis buffer and analyzed by immunoprecipitation with a combination of rabbit anti-VSV and human anti-HIV sera and then by SDS-PAGE.

SDS-PAGE. All samples contained the major VSV structural proteins N and M whether they contained viral glycoprotein. This result is expected because cells infected with the *ts*O45 mutant are known to produce particles lacking G protein (20). The only pellet containing HIV envelope protein was derived from cells transfected with the Env-G 709 construct, the same construct that rescued infectivity. Bands corresponding to both the hybrid precursor and the cleavage product gp120 were observed, although in several experiments we observed only the gp120 band. In addition, a diffuse band which migrated at approximately 35 kDa, the size predicted for the gp41-G hybrid transmembrane subunit, was observed in the Env-G 709 sample. As expected from previous work (25), VSV G protein was seen in the virus pellet when cells were transfected with DNA encoding wild-type G protein.

Because of previous difficulties with shedding of gp120 from virus particles during sucrose gradient sedimentation, we did not analyze the VSV(HIV) pseudotypes by this method. However, the presence of gp120 protein in the viral pellet only after rescue by Env-G 709 almost certainly indicates true incorporation into virus particles since this is the only construct that rescued infectivity; those that did not rescue infectivity (wild-type HIV-1 Env, Env tr-735, and Env G-751) were not seen in the viral pellets.

DISCUSSION

The data that we have presented indicate that the cytoplasmic tail of the VSV G protein can provide a positive signal promoting incorporation of a foreign protein into VSV particles, probably by interacting with VSV nucleocapsid or matrix proteins. The failure of the Env-tr 735 mutant to rescue infectivity indicates that the specific amino acids rather than the length of the cytoplasmic tail are important since this mutant has a truncated tail that is the same length as the VSV G tail in Env-G 709. Our data also indicate that the signal must be located adjacent to the transmembrane domain because the mutant Env-G 751 with the G tail appended to 45 amino acids of the gp41 cytoplasmic domain does not rescue infectivity. Recent data indicate that VSV G probably interacts with the cytoplasmic matrix protein (7). Since the matrix protein itself interacts with the plasma membrane (1), the interaction may require that the G tail be close to the membrane.

Although VSV(HIV) pseudotypes have been described previously (2), these were generated only in cells infected with HIV-1 and wild-type VSV, and the pseudotype particles contained a mixture of both HIV-1 Env and VSV G proteins. In our system, only a specific HIV-1 Env–VSV hybrid protein was incorporated into VSV particles to generate pseudotypes. This is probably because we employed the *ts*O45 mutant, which does not express the VSV G protein on the cell surface at nonpermissive temperatures. A study by Witte and Baltimore (27) of VSV pseudotype formation with another retrovirus, murine leukemia virus, supports this idea. They reported that VSV (murine leukemia virus) pseudotypes form with *ts*O45 at permissive, but not at nonpermissive, temperatures. They also presented evidence that some G protein was required to initiate pseudotype formation before the murine leukemia virus Env protein could be trapped in VSV particles. In our system, the HIV Env-G 709 hybrid protein apparently contains a signal that allows assembly in the absence of VSV G protein. This hybrid protein may interact directly with VSV core proteins or with small G protein fragments that have been detected in *ts*O45 particles lacking G protein (11).

It should be noted that pseudotype formation between VSV *ts*O45 and avian retroviruses at nonpermissive temperatures has been reported (24, 28). This result indicates that pseudotype formation does not always require the presence of wild-type G protein.

Incorporation of the cellular glycoprotein CD4 into VSV *ts*O45 has also been reported previously (21). However, particles containing CD4 were observed only when the experiments were done at the permissive temperature, suggesting that some G protein is required for incorporation of CD4 molecules into VSV. The presence of the VSV G cytoplasmic tail on CD4 did not appear to increase CD4 incorporation into *ts*O45 particles. It is possible that oligomerization influences the interaction of the cytoplasmic domain of VSV G with internal viral proteins (G is a trimer). Therefore, because CD4 is a monomer, the CD4-VSV G hybrid protein (21) may not present the cytoplasmic domain of G in a correct configuration for assembly.

Although our results indicate that the presence of the G cytoplasmic domain is essential for incorporation of a foreign glycoprotein into *ts*O45 VSV particles at nonpermissive temperatures, we have not been able to quantitate the amount of the HIV-VSV G hybrid protein in virus particles relative to the amounts of other VSV proteins. This is especially difficult because the majority of the particles produced during a rescue experiment with *ts*O45 are bald particles lacking any detectable envelope protein (25). An accurate measure of the efficiency with which the hybrid Env-G 709 can substitute for the VSV G protein will likely require development of a system in which specific genes of nonsegmented negative-strand viruses can be replaced and recombinant viruses can be recovered. In such recombi-

nants, the foreign gene could be expressed at the right time and at the proper level relative to the levels of other VSV proteins, allowing a better assessment of its ability to be incorporated into virions.

In addition to providing basic information about viral assembly, the system we have described should prove useful for generating pseudotypes without risk of accidental HIV-1 infection. VSV(HIV) pseudotype production by coinfection with wild-type viruses also generates HIV(VSV) pseudotypes with broad cell tropism. The pure preparations of VSV(HIV) pseudotypes that we have described could provide a safe and rapid assay system for the analysis of molecules that may affect HIV-1 entry.

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