JOURNAL OF VIROLOGY, Feb. 1995, p. 1209–1218 Vol. 69, No. 2
0022-538X/95/$04.00+0

Determinants of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Oligomeric Structure

PANTELIS POUMBOURIOS,[1]* WALID EL AHMAR,[1] DALE A. MCPHEE,[2] AND BRUCE E. KEMP[1]

St. Vincent's Institute of Medical Research, Fitzroy, Victoria 3065,[1] and AIDS Cellular Biology Unit, Macfarlane Burnet Centre for Medical Research, Fairfield, Victoria 3078,[2] Australia

Received 3 March 1994/Accepted 24 October 1994

Oligomerization of the human immunodeficiency virus type 1 envelope (env) glycoproteins is mediated by the ectodomain of the transmembrane glycoprotein gp41. We report that deletion of gp41 residues 550 to 561 resulted in gp41 sedimenting as a monomer in sucrose gradients, while the gp160 precursor sedimented as a mixture of monomers and oligomers. Deletion of the nearby residues 571 to 582 did not affect the oligomeric structure of gp41 or gp160, but deletion of both sequences resulted in monomeric gp41 and predominantly monomeric gp160. Deletion of residues 655 to 665, adjacent to the membrane-spanning sequence, partially dissociated the gp41 oligomer while not affecting the gp160 oligomeric structure. In contrast, deletion of residues 510 to 518 from the fusogenic hydrophobic N terminus of gp41 did not affect the env glycoprotein oligomeric structure. Even though the mutant gp160 and gp120 molecules were competent to bind CD4, the mutations impaired fusion function, gp41-gp120 association, and gp160 processing. Furthermore, deletion of residues 550 to 561 or 550 to 561 plus 571 to 582 modified the antigenic properties of the proximal residues 586 to 588 and the distal residues 634 to 664. Our results indicate that residues 550 to 561 are essential for maintaining the gp41 oligomeric structure but that this sequence and additional sequences contribute to the maintenance of gp160 oligomers. Residues 550 to 561 map to the N terminus of a putative amphipathic α-helix (residues 550 to 582), whereas residues 571 to 582 map to the C terminus of this sequence.

The human immunodeficiency virus type 1 (HIV-1) envelope (env) glycoproteins gp120 and gp41 mediate the attachment of virus particles to the cell surface CD4 receptor and subsequent fusion between the viral envelope, or infected cell surface, and target cell membrane (8, 36, 42, 45, 58). This process allows penetration of the viral genome into host cells.

Posttranslational folding, disulfide bond formation, addition of high-mannose core oligosaccharides, and dimerization of gp160 all occur in the rough endoplasmic reticulum (ER) prior to transport to the Golgi complex for cleavage to gp120 and gp41 (17, 57, 65). The cleaved env glycoproteins are then translocated to the cell membrane for incorporation into budding virions. The gp120-gp41 complex is composed of four gp41 monomers associated noncovalently with three or four gp120 monomers (49, 50, 61).

The gp41 molecule comprises a hydrophobic N terminus (residues 507 to 522, BH8 clone numbering [52]) which mediates membrane fusion (22, 26), a single disulfide-bonded loop (residues 583 to 599) which constitutes an immunodominant antibody epitope (29, 60), a membrane-anchoring sequence (residues 679 to 700) (31, 37), and an extended 150-residue cytoplasmic domain (residues 701 to 851) whose function is unclear (12, 25, 31, 64, 69). Mutagenesis and other approaches suggest that gp120 binding is mediated by three discontinuous gp41 regions involving residues 525 to 532 (C terminal to the fusion peptide) (37), residues 582 to 588 (N terminal to the immunodominant epitope) (3, 5, 43), and residue 634 (N terminal to the membrane-spanning domain) (37).

A feature common to the env glycoproteins of HIV and other enveloped viruses is their oligomeric structure. gp160, gp120, and gp41 appear to have tetrameric structures (14, 49, 50, 56, 61), as do the transmembrane proteins and glycoprotein precursors of HIV-2 and simian immunodeficiency virus (4, 53, 54). Oligomerization of the env glycoproteins of other viruses, such as influenza virus, vesicular stomatitis virus, and Rous sarcoma virus, is a prerequisite for the correct intracellular transport and processing of the glycoproteins (7, 18, 28, 38). While export of HIV-1 gp160 from the ER is essential for the production of mature virion-associated env glycoproteins (66), whether oligomerization is required for transport of the HIV env glycoproteins has not yet been shown.

The oligomerization domains of the HIV-1, HIV-2, and simian immunodeficiency virus env glycoprotein precursors are functionally conserved, since heterodimers of the env glycoprotein precursors form when they are coexpressed in the same cell (10). The relatively conserved gp41 ectodomain is responsible for oligomerization of gp160, since the cytoplasmic and transmembrane domains are not essential (14, 16, 32). Truncation mutagenesis indicates that residues Leu-575 to Leu-636 are important for the oligomerization of gp160, since truncation to Leu-636 does not affect gp160 oligomerization (14, 16, 32) but truncation to Leu-575 drastically reduces gp160 oligomerization efficiency (16). This region partially overlaps with a 33-residue sequence, residues 550 to 582, that has the propensity to form an extended amphipathic α-helix (27). Evidence that this sequence encodes α-helical structure has been inferred from circular dichroism analysis of a synthetic peptide that corresponds to the gp41 sequence (63). This extended α-helical domain contains a heptad repeat of leucine and isoleucine residues, known as a leucine zipper motif, and is conserved in the transmembrane proteins of HIV and several other enveloped viruses (1, 9, 27, 59). Leucine zippers are known to mediate dimerization of several transcription factors (35, 40, 48), and it was suggested that this motif may also mediate dimerization of the HIV env glycoproteins (9, 11, 27). Nonconservative substitutions introduced into the leucine zipper-like motif, however, did not completely disrupt the oligo-

* Corresponding author. Mailing address: St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, VIC 3065, Australia. Phone: 61 3 288 2480. Fax: 61 3 416 2676.

meric structure of the gp160 precursor, although they impaired env glycoprotein fusion function and/or viral infectivity (6, 11).

In the present paper, we report that sequences in the putative α-helical region are essential for gp160 and gp41 oligomeric structure.

MATERIALS AND METHODS

Plasmid constructs. We used the vaccinia virus/T7 RNA polymerase transient expression system (20, 23, 24) for expression of wild-type and mutant env glycoproteins. The expression plasmid pTM.1 was a kind gift of O. Elroy-Stein and B. Moss, and the plasmid pPE16 (15), which contains the full-length *env* gene (BH8 clone [52]), was kindly provided by P. E. Earl, National Institute of Allergy and Infectious Diseases, Bethesda, Md. To enable cloning of the *env* gene into the expression plasmid, pTM.1, an *Nco*I site flanking the ATG initiation codon of the *env* gene was introduced by the oligonucleotide-directed in vitro mutagenesis procedure (Amersham International, plc, Buckinghamshire, United Kingdom). An *Stu*I site was introduced immediately 3′ of the *env* gene TAA stop signal by PCR amplification of a 905-bp fragment (nucleotides 7467 to 8372 [52]) with Pfu polymerase (Stratagene, La Jolla, Calif.), the oligonucleotide pair 5′-CAGAACAAGCTTCTGAGGGCTATT and 3′-CCTTTCCTAAAACGATAT TCCGGAATTATT, and pPE16 as a DNA template. The *env* gene was cloned as *Nco*I-*Bam*HI and *Bam*HI-*Stu*I fragments into pTM.1 to give pTMenv. An *Xba*I-*Eco*RI fragment of pTMenv, containing the first 1,126 bases of the mutated *env* gene, was then cloned into M13mp19 for restoration of Arg at position 2 by in vitro mutagenesis with the oligonucleotide 5′-CTCCTTCACTCTCATGGTAT TATCG to give pTMenv.2. An *Eco*RI-*Sal*I fragment from pTMenv.2 which contains the 1,427 3′ nucleotides of *env* was cloned into M13mp18 for use as a single-stranded template for construction of gp41 mutants by the in vitro mutagenesis procedure described above. The sequences of mutants and PCR products were determined by the dideoxy chain termination procedure with the Sequenase method (U.S. Biochemical, Cleveland, Ohio).

Cells and virus. HeLa-T4 cells, which constitutively express the CD4 receptor, were obtained through the AIDS Research and Reference Reagents Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from P. J. Maddon (44). HeLa-T4 cells were maintained in Dulbecco's minimal essential medium containing 10% fetal calf serum and 1 mM glutamine ($DMEMF_{10}$) with 500 μg of G418 (GIBCO BRL, Gaithersburg, Md.) per ml, while HeLa cells were cultured in $DMEMF_{10}$ alone. The recombinant vaccinia virus vTF7-3, which directs cytoplasmic expression of the bacteriophage T7 RNA polymerase, was obtained through the AIDS Research and Reference Reagents Program, Division of AIDS, National Institute for Allergy and Infectious Diseases, from T. M. Fuerst and B. Moss (23, 24). For expression of wild-type and mutant env glycoproteins, monolayers of 450,000 cells in 9-cm^2 wells (Linbro, McLean, Va.) were infected with vTF7-3 at a multiplicity of infection of 20 PFU per cell for 30 min. Following removal of the virus inoculum, the cells were transfected with 10 μg of plasmid DNA by the lipofectin procedure (GIBCO BRL) (21). The transfection mixture was replaced with $DMEMF_{10}$ at 4 h posttransfection for maintenance of cells until completion of the experiment.

Sucrose density gradient centrifugation. HeLa cells (900,000 in two 9-cm^2 wells) were infected with vTF7-3 and transfected with plasmid DNA as described above. The cells were lysed at 24 h posttransfection with phosphate-buffered saline (PBS) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 for 30 min on ice. Lysates were centrifuged at 10,000 × *g* for 10 min and then layered onto 5 to 20% sucrose gradients containing PBS and 1% Triton X-100. Centrifugation was performed in a Beckman SW41Ti rotor at 35,000 rpm for 19 h at 4°C. Following centrifugation, 0.5-ml fractions were obtained, and each fraction was subjected to trichloroacetic acid (TCA) precipitation and electrophoresis on 3.5 to 15% gradient gels in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions (39). Proteins were transferred to nitrocellulose, immunoblotted with antibodies directed against gp41 and/or gp120, and then probed with radioiodinated protein A. Immunoblots were visualized by autoradiography or by scanning in a Phosphorimager SF (Molecular Dynamics, Sunnyvale, Calif.). Sucrose gradients were calibrated with the markers catalase (11.3S), ^{125}I-thyroglobulin (19.4S), ^{125}I-aldolase (7.3S), and ^{125}I-ovalbumin (3.55S) (Pharmacia, Uppsala, Sweden). Protein A and calibration markers were radioiodinated by the chloramine T procedure (30). To characterize the sedimentation of gp160 and gp41 oligomers, each fraction was cross-linked with 0.5 mM bis(sulfosuccinimidyl)suberate (Pierce Chemical Company, Rockford, Ill.) for 1 h on ice. Samples were then quenched with 100 mM glycine (pH 7.8) for 1 h on ice prior to TCA precipitation, SDS-PAGE, and immunoblotting as described above.

HeLa-T4 syncytium assay. HeLa-T4 cells (350,000 in 9-cm^2 wells) were infected with vTF7-3 and transfected with plasmid DNA as described above. At 24 h posttransfection, cells were washed with PBS and stained by the May-Grünwald-Giemsa technique as described previously (11).

Biosynthetic labeling of env glycoproteins. HeLa cells (450,000 in 9-cm^2 wells) were infected with vTF7-3 and transfected with plasmid DNA as described above. At 19 h posttransfection, cells were starved for cysteine and methionine by incubation in cysteine- and methionine-deficient $DMEMF_{10}$ (ICN, Costa Mesa, Calif.) for 30 min at 37°C. The cells were then pulsed with 200 μCi of Tran-^{35}S-label (ICN) for 20 min, washed with $DMEMF_{10}$, and chased with complete $DMEMF_{10}$ for 5 h at 37°C. Following the chase period, culture supernatants were removed and centrifuged at 10,000 × *g* for 1 min to remove cells that had dissociated from the monolayer. Monolayers were lysed with single-strength lysis buffer (50 mM Tris HCl [pH 7.4] containing 600 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) for 30 min on ice and were pooled with cells that had been pelleted from culture supernatants. Cell lysates were clarified by centrifugation at 10,000 × *g* for 10 min. Culture supernatants were supplemented with a one-third volume of quadruple-strength lysis buffer. The volumes of cell lysates were adjusted to equal the volumes of culture supernatants. In one experiment, infected and transfected cells were pulsed for 17 min at 37°C and either immediately lysed or chased for 5 h with complete medium at 37°C before lysis.

Radioimmunoprecipitation. A one-fifth volume of each cell lysate or culture supernatant was used in radioimmunoprecipitation assays. Samples were precleared overnight with 10% (vol/vol) protein A-agarose (Bio-Rad, Richmond, Calif.) coated with normal human immunoglobulin G (IgG). When monoclonal antibody (MAb) OKT4 was used for immunoprecipitation, cell lysates and culture supernatants were adjusted to pH 8.2 by adding an equal volume of lysis buffer (pH 8.8) prior to preclearing with protein A-agarose coated with irrelevant MAbs of the same isotype as the immunoprecipitating antibody. When MAb 2A2/26 or Chessie 8 was used, beads were precoated with rabbit IgG to mouse IgG (Dakopatts, Glostrup, Denmark). Cleared lysates and supernatants were then incubated with antibodies at 4°C overnight. Antibody-antigen complexes were immunoprecipitated with protein A-agarose for 45 min at room temperature and then washed four times with 50 mM Tris HCl containing 500 mM NaCl, 1 mM EDTA, and 1% Triton X-100 and two times with PBS at the appropriate pH. Immunoprecipitates were then subjected to SDS-PAGE on 4 to 12% Miniprotean gels (Bio-Rad) under reducing conditions followed by autoradiography or Phosphorimager analysis.

For CD4-binding assays, soluble, recombinant CD4 (0.5 μg) was added to precleared cell lysates and culture supernatants. After 1 h, 2 μg of MAb OKT4 was added, and immune complexes were allowed to form overnight at 4°C. Samples were then immunoprecipitated, washed, and subjected to reducing SDS-PAGE and autoradiography as described above.

Antibodies. Antibodies specific for the gp41 immunodominant epitope at residues 588 to 599 [gp41(588-599)] (GIWGCSGKLIC) (29, 60) were purified from pooled HIV-positive human plasma by peptide affinity chromatography with a gp41(588-599) peptide-Sepharose column as described previously (51). Antibodies to gp120 were purified from pooled HIV-positive human plasma by affinity chromatography on a recombinant gp120 (SF2 strain)-Sepharose column as described previously (19). Antibodies specific for the gp41(634-664) sequence, TSLIHSLIEESQNQQEKNEQELLELDKWASL, were purified from human plasma with enzyme-linked immunosorbent assay plates coated with the gp41(634-664) synthetic peptide as described previously (51). The murine MAb H69-67-2A2/26 (MAb 2A2/26) was obtained from Agen Biomedicals Pty. Ltd. (Brisbane, Australia), MAb OKT4 (34) was obtained from the American Type Culture Collection (Rockville, Md.), and Chessie 8 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from George Lewis (41). Murine IgG was purified by affinity chromatography on protein A-Sepharose (Pharmacia).

RESULTS

Effect of deletions on gp160 and gp41 oligomeric structure. Deletion mutations were made in conserved regions of the gp41 ectodomain to determine their role in the env glycoprotein oligomeric structure. The mutants Δ550-561 and Δ571-582 lack 12 N-terminal residues (Leu-550 to Leu-561) and 12 C-terminal residues (Leu-571 to Leu-582) in the putative α-helical region Leu-550 to Leu-582 (27), respectively, as illustrated in Fig. 1. Mutants with deletions in other conserved gp41 domains include Δ510-518, which lacks the hydrophobic fusogenic sequence (22, 26), and Δ655-665, which lacks residues Leu-655 to Trp-665 adjacent to the transmembrane sequence of gp41 and also has the propensity for α-helicity (27). Two mutants that had double deletions, Δ550-561+571-582 and Δ510-518+571-582, were constructed.

The effect of mutations on gp41 and gp160 oligomeric structure was monitored by using two-dimensional sucrose density gradient centrifugation and SDS-PAGE followed by immunoblotting as described previously (14). gp160 was distributed between 19.3S and 5.2S and corresponded to multiple species ranging from tetramer to monomer, respectively, when com-

FIG. 1. Location of mutations in the gp41 ectodomain. Mutations were prepared by the oligonucleotide-directed in vitro mutagenesis procedure. The sequences deleted from gp41 are enclosed in boxes, and the designation of each mutant is indicated in boldface. Lysines in the putative α-helical sequence that were substituted with arginine are indicated by arrowheads. Leucines and isoleucines of the leucine zipper-like motif are in outline type. TM, transmembrane domain.

pared with cross-linked gradients. The predominant species corresponded with the gp160 dimer (Fig. 2) and is consistent with previously published results (14). The peak of untreated gp41 sedimented between 6S and 4.9S, and comparison with cross-linked fractions indicated that the highest-order species in these fractions had a molecular mass of approximately 160 kDa, suggesting that it was tetrameric. This result is also consistent with previously published results that indicate a tetrameric structure for viral gp41 (50, 51, 56). Two minor immunoreactive cross-linked species with molecular masses of approximately 200 kDa (fractions 12 to 16) and 140 kDa (fractions 16 and 17) were observed (indicated by asterisks in Fig. 2). The identities of these species are not known; however, the 200-kDa species may correspond to gp160 monomer complexed with GRP78-BiP (17).

The sedimentation of gp120 with respect to that of gp160 and gp41 is shown in Fig. 3 (W.T. and W.T.-anti-gp120, respectively). While the peaks of gp160 and gp41 sediment to approximately 11S and 5.5S, respectively, gp120 sediments to intermediate fractions, suggesting that it too is oligomeric to some degree. These results are consistent with those of Owens and Compans (49), who have reported that vaccinia virus-expressed gp120 is dimeric, but contrasts with results of other workers which indicate that un-cross-linked gp120 sediments as a monomer in sucrose gradients (14, 61). To define the sedimentation of monomeric gp160 and gp41, lysates of cells expressing wild-type env glycoproteins were boiled in the presence of 1% SDS and 1% β-mercaptoethanol prior to sedimentation. Monomeric gp160 cosedimented with the 7.3S protein aldolase (158 kDa), and monomeric gp41 cosedimented with the 3.55S protein ovalbumin (43 kDa) (Fig. 3, W.T.-SDS/βME TREATED).

Deletion of the 12 N-terminal residues of the putative α-helix, residues 550 to 561, caused gp41 to cosediment with SDS- and β-mercaptoethanol-disrupted monomeric gp41 (Fig. 3, Δ550-561), while the sedimentation of gp160 containing this deletion was skewed towards lower-order oligomeric species. In contrast, deletion of the 12 C-terminal residues of the putative α-helix, residues 571 to 582, did not significantly affect the sedimentation of gp160 or gp41 (Fig. 3, Δ571-582). Deletion of residues 550 to 561 together with residues 571 to 582 resulted in monomeric gp41 and predominantly monomeric gp160 (Fig. 3, Δ550-561+571-578). These results indicate that while deletion of residues 550 to 561 was sufficient to com-

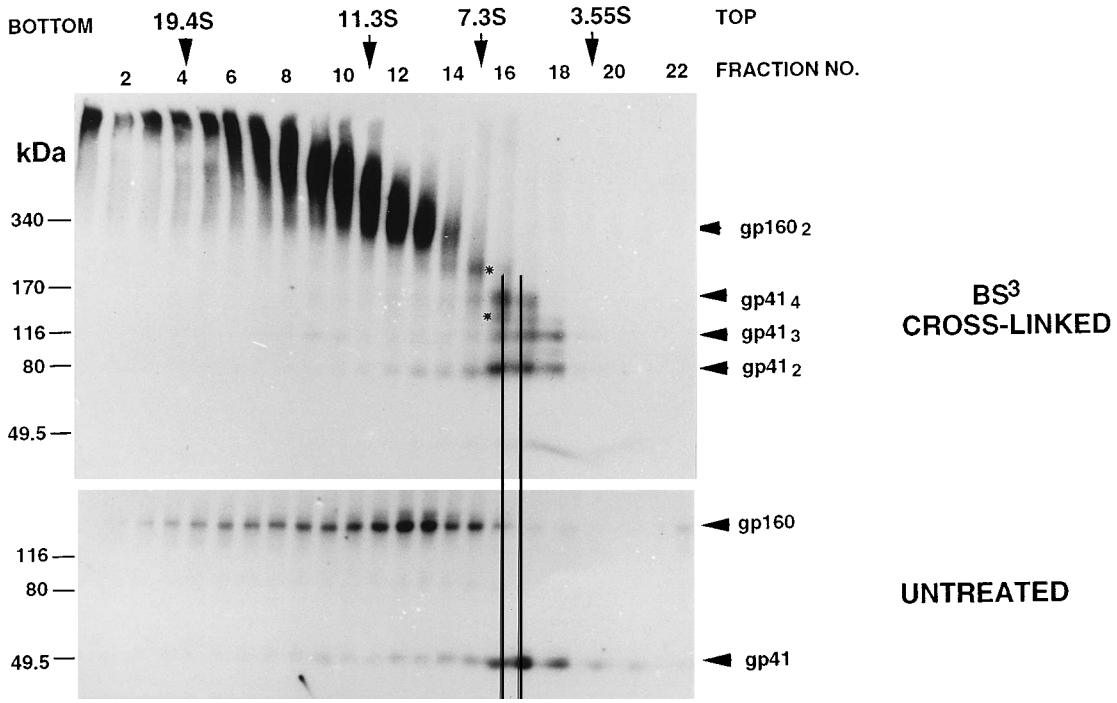

FIG. 2. Cross-linking of env glycoproteins following sedimentation in sucrose density gradients. HeLa cells were infected with vTF7.3 and then transfected with pTMenv.2 for expression of wild-type env glycoproteins. At 24 h posttransfection, cells were lysed, layered onto 5 to 20% sucrose gradients, and centrifuged as described in Materials and Methods. The fractions from one gradient were cross-linked with 0.5 mM bis(sulfosuccinimidyl)suberate (BS^3) for 1 h on ice and then quenched with 100 mM glycine for 1 h on ice. Cross-linked and un-cross-linked fractions were precipitated with TCA and subjected to SDS-PAGE on 3.5 to 15% gradient gels under reducing conditions. Proteins were transferred to nitrocellulose and immunoblotted with an antibody specific for the synthetic peptide gp41(588-599). The sedimentation of the calibration markers ^{125}I-thyroglobulin (19.4S), catalase (11.3S), ^{125}I-aldolase (7.3S), and ^{125}I-ovalbumin (3.55S) is also shown.

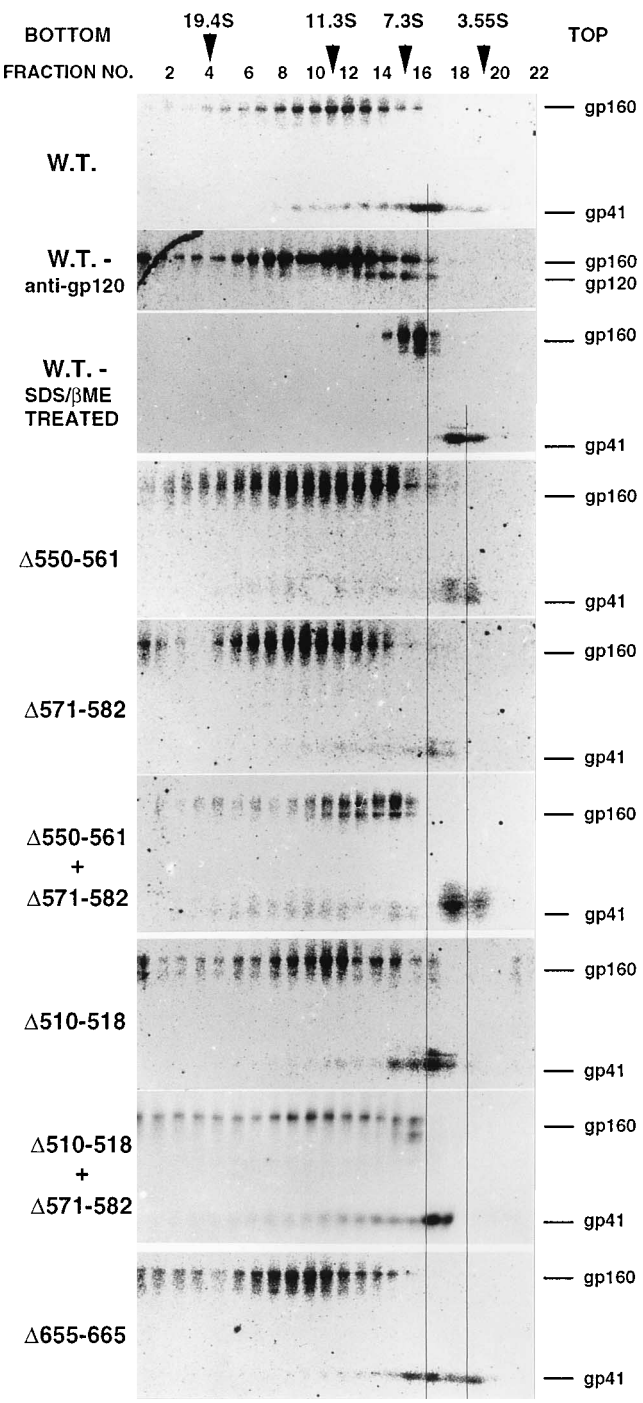

FIG. 3. Sucrose density gradient sedimentation of env glycoproteins bearing mutations in the gp41 ectodomain. vTF7.3-infected HeLa cells were transfected with pTM.1 containing either the wild-type or mutated *env* gene. At 24 h post-transfection, cells were lysed and layered onto 5 to 20% sucrose gradients and centrifuged as described in Materials and Methods. Fractions were precipitated with TCA and subjected to SDS-PAGE on 3.5 to 15% gradient gels under reducing conditions. Proteins were transferred to nitrocellulose and immunoblotted with an antibody specific for the synthetic peptide gp41(588-599) unless indicated otherwise. To observe the sedimentation of gp120, the fractions from one gradient were immunoblotted with an antibody that had been affinity purified from HIV-positive human plasma on recombinant gp120-Sepharose (W.T.-anti-gp120). To locate fractions in which monomeric gp41 and gp160 were present, cell lysates were boiled in the presence of 2.5% SDS and 1% β-mercaptoethanol for 5 min prior to sedimentation (W.T.-SDS/βME TREATED). The sedimentation of calibration standards is indicated at the top.

pletely disrupt the oligomeric structure of gp41, gp160 oligomers were not disrupted unless residues 571 to 582 were also deleted. Deletion Δ550-561 or Δ571-582 led to a marked decrease in the amount of gp160 processed to gp41, but surprisingly, deletion of both regions led to more efficient processing. The gp41 bands observed in Δ550-561 and Δ550-561+571-582 gradients appeared to be more diffuse than those in the other panels of Fig. 3. Deglycosylation of gp160 and gp41 containing the Δ550-561+571-582 mutation with endoglycosidase F resulted in sharp bands with molecular masses of approximately 80 and 28 kDa, which correspond to the gp160 and gp41 core proteins, respectively (data not shown). This result suggests that glycosylation of the mutant glycoproteins may be modified because of glycosyl transferases and trimming enzymes having altered accessibility to glycosylation sites on the mutant monomeric forms in the ER and/or Golgi compartment.

The sedimentation of gp41 and gp160 of mutant Δ510-518 was not altered with respect to the wild type, indicating that the bulky hydrophobic residues of the fusogenic domain are not required for oligomeric structure. Furthermore, deletion of residues 571 to 582 together with residues 510 to 518 did not significantly alter the sedimentation of gp41 and gp160 (Fig. 3, Δ510-518 and Δ510-518+571-582, respectively). For the mutants Δ571-582, Δ510-518, and Δ510-518+571-582, the gp41 peak was in fraction 17 while the wild-type gp41 peak was evenly distributed in fractions 16 and 17 (Fig. 3). These differences in peak distribution were not reproducible. Again, gp160 bearing the double deletion Δ510-518+571-582 was processed more efficiently than was gp160 bearing the Δ571-582 mutation alone.

Gallaher et al. (27) proposed that residues 646 to 665 adjacent to the transmembrane sequence may also form an α-helix. To determine if this region plays a role in maintaining env glycoprotein oligomeric structure, the hydrophobic portion of this putative α-helix, residues 655 to 665, was deleted. This mutation caused gp41 to partially dissociate, with approximately 65% tetramer and 35% monomer, while not affecting the sedimentation of gp160 (Fig. 3, Δ655-665). While residues 655 to 665 appear to contribute to maintaining the gp41 oligomeric structure, this sequence is expendable in the case of gp160.

Recognition of env glycoproteins by MAbs and affinity-purified sequence-specific antibodies. Previous studies on oligomeric viral envelope glycoproteins indicate that the antigenic features of the oligomer are distinct from those of the monomer (7, 13, 28, 47, 50, 51, 68). Earl et al. (17) have shown that gp160 takes up a CD4-binding-competent structure with a $t_{1/2}$ of 15 min postsynthesis, while the $t_{1/2}$ of gp160 dimerization is 30 min. To obtain biosynthetically labeled, preassembled gp160 monomer, cells transfected with the wild-type plasmid were pulsed with [^{35}S]Met/Cys for 17 min and then lysed immediately. Alternatively, pulse-labeled cells transfected with wild-type or mutant plasmids were chased for 5 h in complete medium to allow oligomerization and transport of the glycoproteins prior to lysis and immunoprecipitation with the various antibodies. We used this approach to assess whether deletions affecting the oligomeric structure of gp160 or gp41 also affected its antigenic structure. Four gp41-specific antibodies were compared for their abilities to immunoprecipitate the preassembled and assembled wild-type and pulse-chased mutant glycoproteins. The control MAb, Chessie 8, which has been mapped to a linear epitope in the cytoplasmic domain of gp41 (41), immunoprecipitated all samples to similar levels, except for the pulsed wild type, for which the level was slightly lower (Fig. 4). An antibody specific for residues 634 to 664 was affinity purified from HIV-positive human plasma by using the

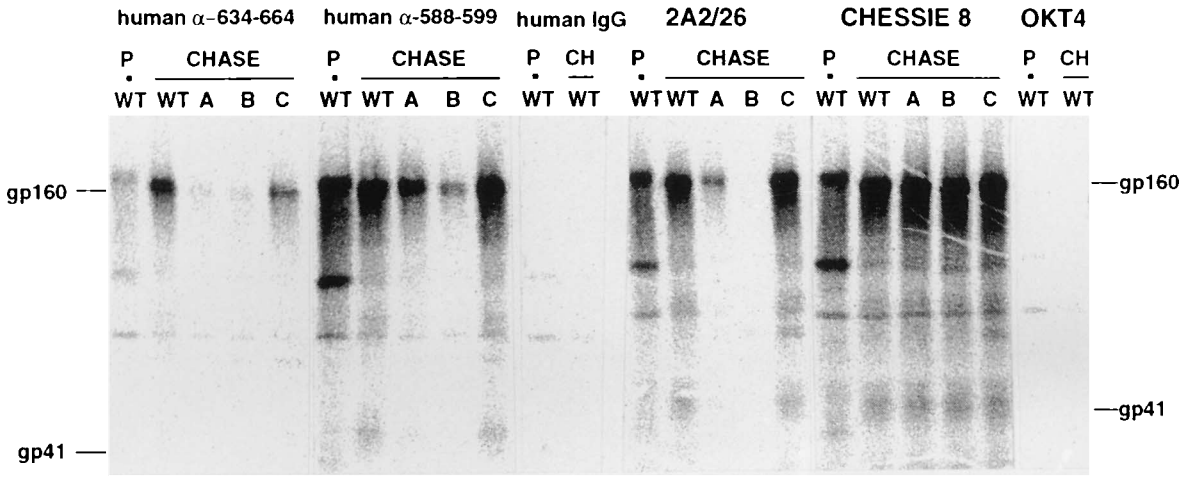

FIG. 4. Recognition of env glycoproteins by MAbs and affinity-purified sequence-specific antibodies. vTF7.3-infected HeLa cells were transfected with pTM.1 containing either the wild-type or mutated *env* gene. At 19 h posttransfection, cells were metabolically labeled for 17 min and either immediately lysed or chased for 5 h with complete medium and then lysed. Cell lysates were precleared with protein A-Sepharose coated with preimmune rabbit IgG for immunoprecipitation with mouse monoclonal IgG bound to protein A-Sepharose coated with rabbit anti-mouse IgG. Alternatively, cell lysates were precleared as described above for immunoprecipitations with protein A-Sepharose coated with affinity-purified human IgG. The immunoprecipitated biosynthetically labeled env glycoproteins were analyzed by SDS-PAGE and autoradiography. P, pulse; CH, chase; WT, wild-type; A, Δ550-561; B, Δ550-561+571-582; C, Δ510-518; human IgG, HIV-negative human IgG.

synthetic peptide gp41(634-664) coated on enzyme-linked immunosorbent assay plates as an affinity support. This antibody, human α-634-664, strongly recognized cross-linked, oligomeric gp41 and gp160 but weakly recognized monomeric env glycoproteins in an immunoblot assay, indicating that its epitope depends on the oligomeric glycoprotein structure for optimal binding (data not shown). Figure 4 shows that human α-634-664 reacted weakly with pulsed wild-type gp160 (monomer) while reacting strongly with pulse-chased wild-type gp160 (oligomer) in the immunoprecipitation assay. The pulse-chased mutant glycoproteins Δ550-561 and Δ550-561+571-582 were immunoprecipitated at low levels, similar to the case for the pulsed wild type, indicating that the conformation at residues 634 to 664 of the mutants resembled that of the preassembled wild-type gp160 rather than that of the assembled wild type. However, lack of recognition by human α-634-664 did not strictly correlate with loss of env glycoprotein oligomeric structure. The Δ550-561 mutant gp160, which is partially oligomeric, was recognized very weakly by this antibody, as was Δ550-561+571-582 gp160, which is almost completely monomeric. Furthermore, Δ510-518 mutant gp160, which does have an oligomeric structure, was immunoprecipitated to levels lower than those for the assembled wild type, indicating that deletion of the fusion domain, while not disrupting the gp160 and gp41 oligomeric structure, promotes a preassembled conformation at the distal 634-664 site. The human α-588-599 antibody did not distinguish between the preassembled and assembled wild-type gp160 or the Δ510-518 mutant. However, compared with the wild type, there was either a slight reduction (Δ550-561) or a marked reduction (Δ550-561+571-582) in recognition by the human α-588-599 antibody for deletions that affected the oligomeric structure. MAb 2A2/26 immunoprecipitated the assembled wild-type and the Δ510-518 mutant glycoproteins to similar levels but exhibited slightly decreased binding to pulsed, preassembled wild type, greatly diminished binding to Δ550-561, and no binding to the Δ550-561+571-582 mutant. The epitope of MAb 2A2/26 is on the N-terminal side of the gp41 immunodominant region and maps to residues 574 to 596 (51). Neither gp160 nor gp41 molecules bearing the deletion Δ586-588 are immunoprecipitated by MAb 2A2/26 (data not shown). The Δ550-561 and Δ550-561+571-582 mutations alter the conformation of the immunodominant epitope such that it is distinct from those of assembled and preassembled wild-type env glycoproteins. Significantly, the level of recognition by MAb 2A2/26 of the gp160 mutants Δ550-561 and Δ550-561+571-582 correlated with the amount of oligomeric gp160 present in sucrose gradients for these mutants (Fig. 3).

To determine if the deletions caused alterations in env glycoprotein function, we examined their effects on glycoprotein fusion function, CD4 binding, and gp120-gp41 association.

Effect of deletion mutations on env glycoprotein fusion function. Figure 5 shows that while the wild-type env glycoprotein induced syncytia with up to 20 to 50 nuclei, all deletion mutants failed to induce syncytia in HeLa-T4 cells, indicating a complete loss of env glycoprotein fusion function. Several studies have shown that fusion function is susceptible to mutations that do not affect other properties of the env glycoproteins, such as gp120-gp41 association, gp160 processing, cell surface expression, CD4 binding, and CD4-induced shedding of gp120 (3, 5, 11, 37). To assess the sensitivity of fusion function to conservative mutations in the putative α-helix, two point mutants with double Lys→Arg substitutions, K569/583→R and K650/660→R, were prepared. The K569/583→R mutation in the putative α-helix resulted in markedly diminished fusion function. In contrast, the K650/660→R mutant was able to produce syncytia that were indistinguishable from the wild type. These results indicate that although the positive charge is maintained at positions 569 and 583, there is a strict requirement for lysine at these positions for fusion function.

Effect of deletions on CD4-binding function and gp120-gp41 association. Loss of fusion function could result from the deletions if the mutant env glycoproteins were unable to bind to CD4. The abilities of biosynthetically labeled env glycoproteins to coimmunoprecipitate with soluble CD4 and MAb OKT4 were tested. Similar levels of cell-associated gp160 were coimmunoprecipitated with soluble, recombinant CD4 for all mutants (Fig. 6A), indicating that the CD4-binding ability of gp160 was unaffected by the deletion mutations in the gp41 domain. Levels of coimmunoprecipitation of cell-associated

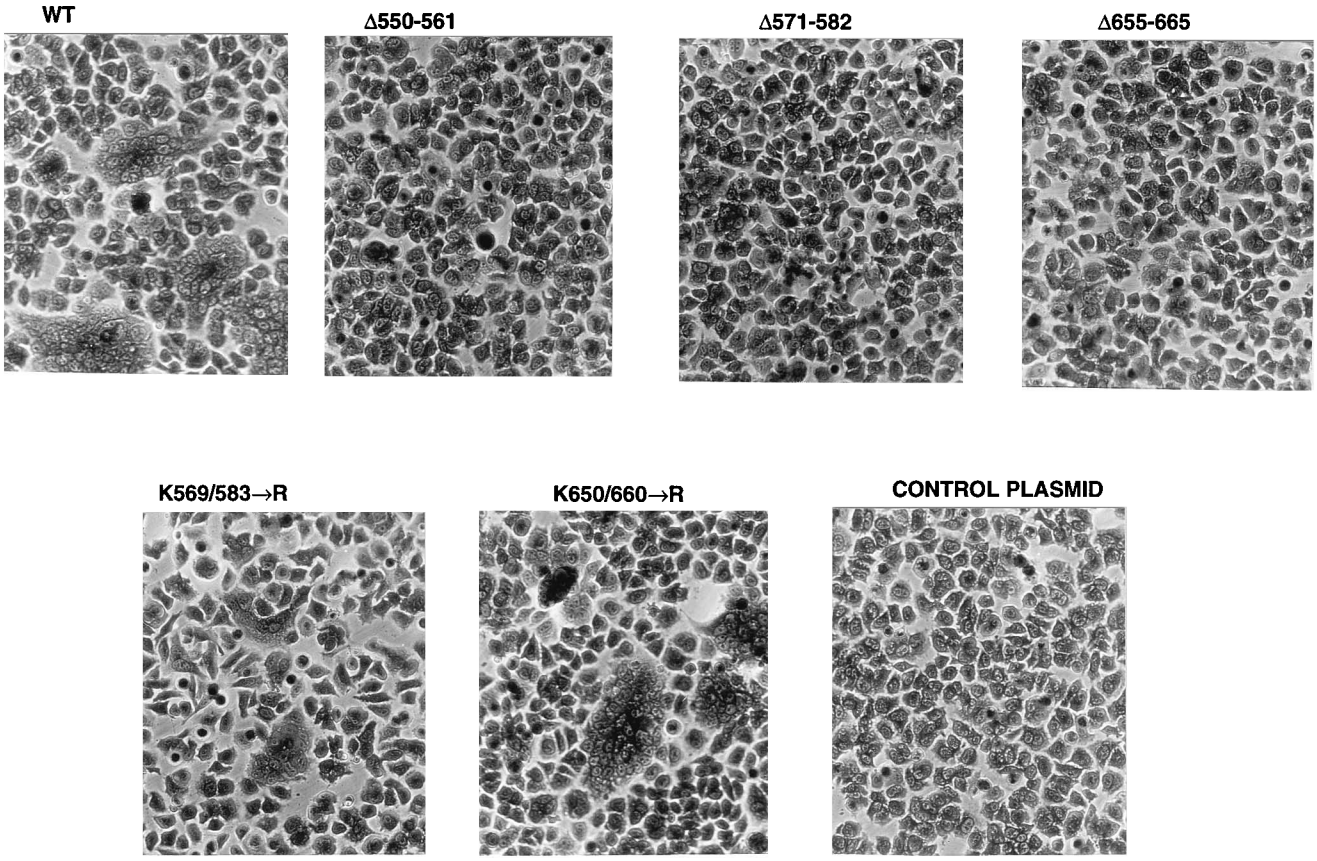

FIG. 5. Effect of gp41 mutations on fusion function. vTF7.3-infected HeLa-T4 cells were transfected with pTM.1 containing either the wild-type (WT) or mutated *env* gene. At 24 h posttransfection, cells were stained for syncytia by the May-Grünwald-Giemsa technique. pTM.1 was used in control transfections.

env glycoproteins bearing the K569/583→R and K650/660→R mutations were also similar to that of the wild type (data not shown).

Results from the CD4-binding assay indicated that little or no gp120 was coimmunoprecipitated with CD4 in the case of all deletion mutants, suggesting that the mutations had decreased the ability of gp41 to anchor gp120. Pulse-chased cell lysates and culture supernatants were immunoprecipitated with a polyclonal anti-gp120 antibody, and the amount of gp120 associated with the cell was compared with that shed into culture supernatants. Figure 6B indicates that approximately 60% of wild-type gp120 is cell associated, while 40% is shed. All deletion mutations led to almost all of the gp120 being shed, irrespective of whether they affected oligomerization. In the case of the Δ655-665 mutant, 5 to 10% of gp120 remained cell associated, suggesting that this mutation had the least effect on gp120 retention. In contrast, the ratio of cell-associated to shed gp120 for the Lys→Arg mutants K569/583→R and K650/660→R was similar to that for the wild-type.

DISCUSSION

Our studies with HIV-1 env glycoproteins bearing deletions in the gp41 ectodomain indicate that residues 550 to 561 are essential for maintaining gp41 and contribute to the gp160 oligomeric structure. While deletion of the nearby residues 571 to 582 alone did not affect the sedimentation of gp41 or gp160 in sucrose gradients, deletion of both domains led to almost complete disruption of the oligomeric structure of gp160. Deletion of residues 655 to 665, which are adjacent to the transmembrane domain, partially disrupted gp41 oligomers while not affecting gp160. In contrast, deletion of residues 510 to 518 from the fusion domain did not affect gp160 or gp41 oligomeric structure.

Residues Leu-550 to Leu-561 and Leu-571 to Leu-582 map to the N and C termini, respectively, of the putative amphipathic α-helical/leucine zipper-like domain of gp41. Leucine zippers are characterized by a repeat of hydrophobic residues spaced every four and then three residues apart such that they fall on the same face of an amphipathic α-helix. Dimerization of the bZIP class of transcription factors occurs through hydrophobic interactions between the leucine zippers of two monomers (48), and the analogous sequences present in HIV-1 gp41 and a number of other viral transmembrane proteins have been postulated to mediate their dimerization (1, 9, 27, 59). The leucine zipper-like motif is conserved among HIV-1 isolates; however, Ile or Val is substituted for Leu in some positions, while more extensive substitutions occur in the analogous regions of the HIV-2 and simian immunodeficiency virus transmembrane glycoproteins (46).

Our finding that deletion of the N-terminal but not the C-terminal portion of the gp41 α-helical/leucine zipper-like sequence effected disruption of the env glycoprotein oligomeric structure is not consistent with a model for gp41 oligomerization involving simply hydrophobic interactions throughout the entire leucine zipper as occurs in the bZIP class of transcription factors (48). It has been proposed that the putative α-helix of HIV-1 gp41 may be analogous to the 54-

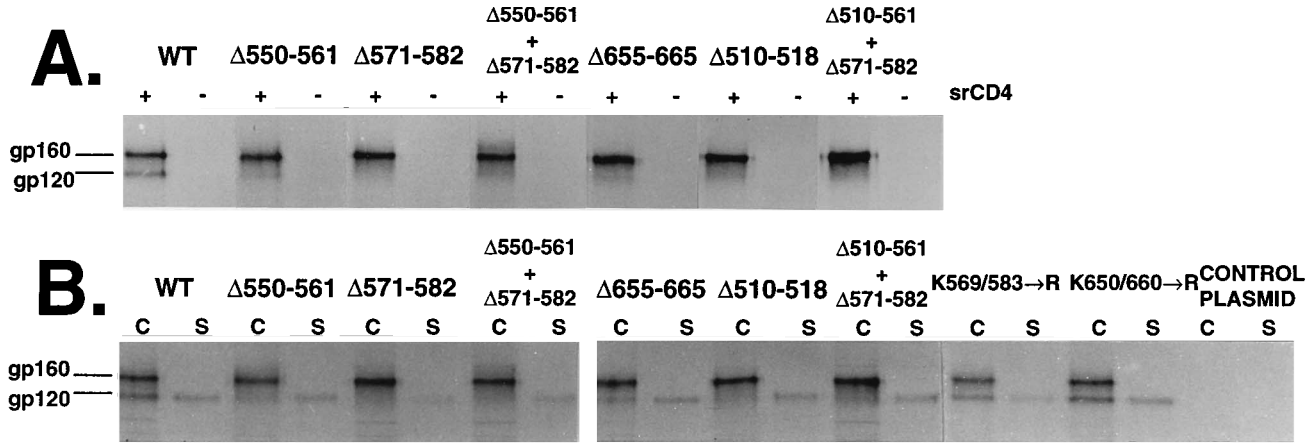

FIG. 6. (A) Effect of gp41 mutations on CD4 binding. vTF7.3-infected HeLa cells were transfected with pTM.1 containing either the wild-type (WT) or mutated *env* gene. At 19 h posttransfection, cells were metabolically labeled for 20 min and then chased with complete medium for 5 h before lysis. Soluble, recombinant CD4 (srCD4) (0.5 μg) was added to precleared cell lysates, and gp160-CD4 complexes were coimmunoprecipitated with MAb OKT4 and protein A-agarose (+). srCD4 was excluded from control immunoprecipitations (−). Labeled env glycoproteins that coimmunoprecipitated with srCD4 and OKT4 were visualized following SDS-PAGE on 4 to 12% gradient gels under reducing conditions and autoradiography. (B) Effect of mutations on gp41-gp120 association. Aliquots of cell lysates (C) and culture supernatants (S) obtained for panel A were precleared with protein A-agarose coated with normal human IgG and then incubated with antibody that had been affinity purified from HIV-positive human plasma on recombinant gp120-Sepharose. Immune complexes were then immunoprecipitated with protein A-agarose, and proteins were analyzed by SDS-PAGE and autoradiography as described for panel A. pTM.1 was used in control transfections.

residue extended amphipathic α-helix of the influenza virus hemagglutinin transmembrane protein HA2 (27). The trimeric structure of hemagglutinin is stabilized primarily by hydrophobic intermonomer interactions in the N-terminal half of the extended helix, while ionic and polar intermonomer interactions occur in the C-terminal half of each helix (67). Hydrophobic residues in the N terminus of the gp41 putative amphipathic α-helix may make analogous interactions in twofold or fourfold symmetry to stabilize the gp41 oligomer. Close inspection of data presented by Chen and coworkers (6) indicates that substitution of Ile-559 (Ile-554 in the BH8 clone numbering) by proline results in bimodal sedimentation of gp160, with approximately 25 to 30% cosedimenting with monomeric gp120. These results indicate that oligomer destabilization may be effected by a nonconservative point mutation in the 550-561 sequence. Although prolines are known to break α-helices, Heinz et al. (33) recently reported that a histidine-proline insertion in an α-helix of T4 lysozyme, while inducing a "looping out" of the α-helix, was tolerated by the global structure. The recent elucidation of the three-dimensional structure of a fragment derived from the low-pH-activated form of hemagglutinin (2) shows that while a marked structural change occurs following low-pH activation, the N-terminal portion of the extended amphipathic α-helix is the only part of the structure that is not altered. These residues form part of the triple-stranded coiled coil in both the pH 7- and low-pH-activated forms and provide trimer stabilization. This raises the possibility that the 550-561 sequence may also play a stabilization role for the fusion-activated form of gp41. The importance of the 550-561 sequence in glycoprotein function is highlighted in a previous study that shows that mutation of Leu-550 or Leu-561 (BH8 numbering) to glycine markedly decreases gp120-gp41 association and gp160 processing and abolishes fusion function (3).

Deletion of residues 550 to 561 was sufficient to completely disrupt gp41 oligomers to monomers but not gp160 oligomers, suggesting that additional sequences mediate gp160 oligomerization as well as maintenance of oligomeric structure. One candidate domain includes residues 571 to 582, which when deleted together with residues 550 to 561 lead to almost complete loss of the gp160 oligomeric form. A second candidate domain may encompass residues 655 to 665, which are adjacent to the membrane-spanning domain and have been postulated to form part of an α-helix (27). Deletion of these residues led to partial disruption of gp41 oligomers while having no observable effect on gp160 oligomeric structure. One interpretation of this result is that the gp41 oligomer is intrinsically less stable than gp160 and that deletion of a minor assembly domain may result in an observable effect on gp41 but not gp160 oligomeric structure. However, the 655-665 sequence is C terminal to Leu-636; all of the sequence required for gp160 oligomeric structure is N terminal to this residue (14, 16, 32). Intermonomer contacts between gp160 (and gp41) sequences that are N terminal to Leu-636 may confer the highest level of oligomer stability and therefore override destabilization of the oligomer resulting from deletion of residues 655 to 665. Our results therefore suggest that HIV-1 env glycoprotein oligomerization is mediated by multiple domains. A precedent for this idea is provided by the influenza virus hemagglutinin three-dimensional structure. While the N-terminal half of the extended amphipathic α-helix provides the most crucial stabilizing intermonomer contacts, other domains, including an extended chain in HA2, a β loop in HA1, and an oligosaccharide moeity that spans the interface at the membrane-distal globular head, confer additional oligomer stability (67).

Significantly, gp160 bearing the Δ550-561 and Δ550-561+571-582 mutations was processed, although less efficiently than the wild type, which suggests that these mutants are transported from the ER to the trans-medial Golgi compartment for cleavage (57, 65). Since viral env glycoprotein oligomerization appears to be a prerequisite for intracellular transport (7, 18, 28, 38), it is likely that these mutants assemble for initial export from the ER but that their oligomeric structure is lost subsequently. The 550-561 and 571-582 sequences are therefore essential for maintenance of the oligomeric structure but may not be essential for the formation of a transportable oligomer.

The question of whether disruption of env glycoprotein oligomeric structure resulting from the Δ550-561 or Δ550-561+571-582 mutation was due to the deletion of oligomer-

ization domains or due to global conformational changes that cause the loss of oligomeric structure cannot be answered without direct three-dimensional structural information. Indeed, structural differences between monomeric and oligomeric forms of the influenza virus hemagglutinin (7, 28, 47, 68) as well as HIV-1 gp160 and gp41 (13, 50, 51) have been detected by using polyclonal antibodies and MAbs. Our results indicate that disruption of oligomeric structure resulting from the deletions is also accompanied by alterations to antigenic structure. Comparison of the antigenic properties of the Δ550-561 and Δ550-561+571-582 mutants with those of the preassembled and assembled wild-type glycoproteins suggested that the mutants resembled the preassembled wild-type glycoprotein at residues 634 to 664. Significantly, deletion of the hydrophobic fusion domain did not affect the oligomeric structure of gp160 or gp41 but induced a monomer-like conformation at residues 634 to 664, suggesting that alterations at residues 634 to 664 are not strictly dependent on loss of oligomeric structure. The homologous fusion domain of the influenza virus hemagglutinin is wrapped around the fibrous core of HA2 which is formed by the packing of the extended amphipathic α-helices (67). By analogy, deletion of bulky hydrophobic amino acids from the gp41 fusogenic sequence would introduce a cavity in the hydrophobic core of the tetramer. While insufficient to disrupt the oligomer, this deletion may nevertheless destabilize the oligomeric structure so that a conformational change at the distal residues 634 to 664 results. In contrast, the mutants were antigenically distinct from both the wild-type preassembled monomer and assembled oligomer at residues 588 to 599, suggesting that the Δ550-561 and Δ550-561+571-582 mutations may have induced proximal conformational effects at this site. The Δ655-665 mutant glycoproteins were recognized to the same levels as the assembled wild-type glycoproteins (data not shown), indicating that conformational alterations at residues 588 to 599 do not necessarily accompany the loss of oligomeric structure.

All deletions abolished fusion function, decreased gp160 precursor cleavage, and abolished or greatly diminished the ability of gp41 to anchor gp120, but only three deletions (Δ550-561, Δ550-561+571-582, and Δ655-665) affected the oligomeric structure of the glycoproteins, while none affected CD4-binding function. It appears from data presented here and elsewhere (3, 5, 11, 37) that the earlier a function is acquired during the folding-assembly-maturation pathway, the more resistant that function is to mutation. CD4-binding competence and oligomerization occur relatively early and are more resistant to mutation than is precursor cleavage and consequent association between gp120 and gp41, which occurs later in the Golgi compartment (17), which may reflect the level of structural complexity required for each function. Fusion function was especially sensitive to potential structural changes in the putative α-helix/leucine zipper, since the substitutions K569/583→R resulted in a marked decrease in fusogenic potential without affecting gp160 and gp41 oligomerization (data not shown), gp41-gp120 association, and gp160 cleavage. These substitutions are conservative and are expected to maintain potential electrostatic interactions with Glu-579 or Asp-584 in this region; however, the sizes and shapes of the two amino acids are different, as are their pK_a values. Replacement of Lys with Arg may alter the nature of potential electrostatic interactions or, alternatively, introduce structural perturbations in the helical region that compromise the ability of gp41 to undergo the correct conformational changes that precede membrane fusion (2, 55, 62). Lysine at residue 569 is conserved in HIV-1 isolates, while Lys, Arg, or in some cases Gln is present at residue 583, suggesting that the former position may be the more important of the two (46).

Our results support the concept that the 33-residue putative amphipathic α-helical/leucine zipper-like sequence of gp41 is important in maintenance of HIV-1 env glycoprotein oligomeric structure. This motif is conserved in the transmembrane proteins of retroviruses, raising the possibility that it also has a common structural role.

ACKNOWLEDGMENTS

We are grateful to Frosa Katsis for the preparation of oligonucleotides.

This work was supported by the National Health and Medical Research Council and the Commonwealth AIDS Research Grants Program. P.P. is a CARG research fellow, and B.E.K. is an NHMRC research fellow.

REFERENCES

1. **Buckland, R., and F. Wild.** 1989. Leucine zipper motif extends. Nature (London) **338:**547.
2. **Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley.** 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature (London) **371:**37–43.
3. **Cao, J., L. Bergeron, E. Helseth, M. Thali, H. Repke, and J. Sodroski.** 1993. Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. J. Virol. **67:**2747–2755.
4. **Chakrabarti, S., T. Mizukami, G. Franchini, and B. Moss.** 1990. Synthesis, oligomerization, and biological activity of the human immunodeficiency virus type 2 envelope glycoprotein expressed by a recombinant vaccinia virus. Virology **178:**134–142.
5. **Chen, S. S.-L.** 1994. Functional role of the zipper motif region of human immunodeficiency virus type 1 transmembrane protein gp41. J. Virol. **68:** 2002–2010.
6. **Chen, S. S.-L., C.-N. Lee, W.-R. Lee, K. McIntosh, and T.-H. Lee.** 1993. Mutational analysis of the leucine zipper-like motif of the human immunodeficiency virus type 1 envelope transmembrane glycoprotein. J. Virol. **67:** 3615–3619.
7. **Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius.** 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. J. Cell Biol. **103:**1179–1191.
8. **Dalgleish, A. G., P. C. L. Beverly, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312:** 763–767.
9. **Delwart, E. L., and G. Mosialos.** 1990. Retroviral envelope glycoproteins contain a "leucine zipper"-like repeat. AIDS Res. Hum. Retroviruses **6:**703–706.
10. **Doms, R. W., P. L. Earl, S. Chakrabarti, and B. Moss.** 1990. Human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus *env* proteins possess a functionally conserved assembly domain. J. Virol. **64:** 3537–3540.
11. **Dubay, J. W., S. J. Roberts, B. Brody, and E. Hunter.** 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. J. Virol. **66:**4748–4756.
12. **Dubay, J. W., S. J. Roberts, B. H. Hahn, and E. Hunter.** 1992. Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. J. Virol. **66:**6616–6625.
13. **Earl, P. L., C. C. Broder, D. Long, S. A. Lee, J. Peterson, S. Chakrabarty, R. W. Doms, and B. Moss.** 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. **68:**3015–3026.
14. **Earl, P. L., R. W. Doms, and B. Moss.** 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc. Natl. Acad. Sci. USA **87:**648–652.
15. **Earl, P. L., A. W. Hugin, and B. Moss.** 1990. Removal of cryptic poxvirus transcription termination signals from the human immunodeficiency virus type 1 envelope gene enhances expression and immunogenicity of a recombinant vaccinia virus. J. Virol. **64:**2448–2451.
16. **Earl, P. L., and B. Moss.** 1993. Mutational analysis of the assembly domain of the HIV-1 envelope glycoprotein. AIDS Res. Hum. Retroviruses **9:**589–594.
17. **Earl, P. L., B. Moss, and R. W. Doms.** 1991. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J. Virol. **65:**2047–2055.

18. **Einfeld, D., and E. Hunter.** 1988. Oligomeric structure of a prototype retrovirus glycoprotein. Proc. Natl. Acad. Sci. USA **85:**8688–8692.
19. **El Ahmar, W., P. Poumbourios, D. A. McPhee, and B. E. Kemp.** 1991. N-terminal residues 105–117 of HIV-1 gp120 are not involved in CD4 binding. AIDS Res. Hum. Retroviruses **7:**855–858.
20. **Elroy-Stein, O., T. R. Fuest, and B. Moss.** 1989. Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. Proc. Natl. Acad. Sci. USA **86:**6126–6130.
21. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA **84:**7413–7417.
22. **Freed, E. O., D. J. Myers, and R. Risser.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc. Natl. Acad. Sci. USA **87:**4650–4654.
23. **Fuerst, T. R., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7:**2538–2544.
24. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
25. **Gabuzda, D. H., A. Lever, E. Terwilliger, and J. Sodroski.** 1992. Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoproteins. J. Virol. **66:**3306–3315.
26. **Gallaher, W. R.** 1987. Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. Cell **50:**327–328.
27. **Gallaher, W. R., J. M. Ball, R. F. Garry, M. C. Griffin, and R. C. Montelaro.** 1989. A general model for the transmembrane proteins of HIV and other retroviruses. AIDS Res. Hum. Retroviruses **5:**431–440.
28. **Gething, M.-J., K. McCammon, and J. Sambrook.** 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell **46:**939–950.
29. **Gnann, J. W., Jr., J. A. Nelson, and M. B. A. Oldstone.** 1987. Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. J. Virol. **61:**2639–2641.
30. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89:**114–123.
31. **Haffar, O. K., D. J. Dowbenko, and P. W. Berman.** 1988. Topogenic analysis of the human immunodeficiency virus type 1 envelope glycoprotein, gp160, in microsomal membranes. J. Cell Biol. **107:**1677–1687.
32. **Hallenberger, S., S. P. Tucker, R. J. Owens, H. B. Bernstein, and R. W. Compans.** 1993. Secretion of a truncated form of the human immunodeficiency virus type 1 envelope glycoprotein. Virology **193:**510–514.
33. **Heinz, D. W., W. A. Baase, F. W. Dahlquist, and B. W. Mattews.** 1993. How amino acid insertions are allowed in an α-helix of T4 lysozyme. Nature (London) **361:**561–564.
34. **Hoffman, R. A., P. C. Kung, W. P. Hansen, and G. Goldstein.** 1980. Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. Proc. Natl. Acad. Sci. USA **77:**4914–4917.
35. **Hu, J. C., E. K. O'Shea, P. S. Kim, and R. T. Sauer.** 1990. Sequence requirements for coiled-coils: analysis with λ repressor-GCN4 leucine zipper fusions. Science **250:**1400–1403.
36. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312:**767–768.
37. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237:**1351–1355.
38. **Kreis, T. E., and H. F. Lodish.** 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. Cell **46:**929–937.
39. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
40. **Landschultz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:**1759–1764.
41. **Lewis, G., Y. Abacioglu, T. Fouts, J. Samson, M. Moorman, G. Bauer, R. Tuskan, G. Cole, and R. Kamin-Lewis.** 1991. Epitope dominance in the antibody response to recombinant gp160 of HIV-IIIB, p. 157–163. *In* F. Brown, R. Chanock, H. S. Ginsberg, and R. Lerner (ed.), Vaccines 91. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engelman.** 1986. Induction of CD4-dependent cell fusion by the HTLV-111/LAV envelope glycoprotein. Nature (London) **323:**725–728.
43. **Lopalco, L., R. Longhi, F. Ciccomascolo, A. DeRossi, M. Pelagi, F. Andronico, J. P. Moore, T. Schultz, A. Beretta, and A. G. Siccardi.** 1993. Identification of human immunodeficiency virus type 1 glycoprotein gp120/gp41 interacting sites by the idiotypic mimicry of two monoclonal antibodies. AIDS Res. Hum. Retroviruses **9:**33–39.
44. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
45. **McDougal, J. S., J. K. A. Nicholson, G. D. Cross, S. P. Cort, M. S. Kennedy, and A. C. Mawle.** 1986. Binding of the human retrovirus HTLV-III/LAV/ARV/HIV to the CD4 (T4) molecule: conformation dependence, epitope mapping, antibody inhibition and potential for idiotype mimicry. J. Immunol. **137:**2937–2944.
46. **Myers, G., B. Korber, S. Wain-Hobson, R. F. Smith, and G. N. Pavlakis.** 1993. Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
47. **Nestorowicz, A., G. Laver, and D. C. Jackson.** 1985. Antigenic determinants of influenza virus haemagglutinin. X. A comparison of the physical and antigenic properties of monomeric and trimeric forms. J. Gen. Virol. **66:**1687–1695.
48. **O'Shea, E. K., J. D. Klemm, P. S. Kim, and T. Alber.** 1991. X-ray structure of the GCN4 leucine zipper, a two stranded, parallel coiled coil. Science **254:**539–544.
49. **Owens, R. J., and R. W. Compans.** 1990. The human immunodeficiency virus type 1 envelope glycoprotein precursor acquires aberrant intermolecular disulfide bonds that may prevent normal proteolytic processing. Virology **179:**827–833.
50. **Pinter, A., W. J. Honnen, S. A. Tilley, C. Bona, H. Zaghouani, M. K. Gorny, and S. Zolla-Pazner.** 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J. Virol. **63:**2674–2679.
51. **Poumbourios, P., D. A. McPhee, and B. E. Kemp.** 1992. Antibody epitopes sensitive to the state of human immunodeficiency virus type 1 gp41 oligomerization map to a putative α-helical region. AIDS Res. Hum. Retroviruses **8:**2055–2062.
52. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Gharayeb, N. T. Chang, R. C. Gallo, and F. Wong-Stahl.** 1984. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
53. **Rey, M.-A., B. Krust, A. G. Laurent, L. Montagnier, and A. G. Hovanessian.** 1989. Characterization of human immunodeficiency virus type 2 envelope glycoproteins: dimerization of the glycoprotein precursor during processing. J. Virol. **63:**647–658.
54. **Rey, M.-A., A. G. Laurent, J. McClure, B. Krust, L. Montagnier, and A. G. Hovanessian.** 1990. Transmembrane envelope glycoproteins of human immunodeficiency virus type 2 and simian immunodeficiency virus SIV-mac exist as homodimers. J. Virol. **64:**922–926.
55. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174:**407–415.
56. **Schawaller, M., G. E. Smith, J. J. Skehel, and D. C. Wiley.** 1989. Studies with crosslinking reagents on the oligomeric structure of the *env* glycoprotein of HIV. Virology **172:**367–369.
57. **Stein, B. S., and E. G. Engleman.** 1990. Intracellular processing of the gp160 HIV-1 envelope precursor. J. Biol. Chem. **265:**2640–2649.
58. **Stein, B. S., S. D. Gouda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engelman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.
59. **Tucker, S. P., R. V. Srinivas, and R. W. Compans.** 1991. Molecular domains involved in oligomerization of the Friend murine leukemia virus envelope glycoprotein. Virology **185:**710–720.
60. **Wang, J. J. G., S. Steel, R. Wisniewolski, and C. Y. Wang.** 1986. Detection of antibodies to human T-lymphotropic virus type III by using a synthetic peptide of 21 amino acid residues corresponding to a highly antigenic segment of gp41 envelope protein. Proc. Natl. Acad. Sci. USA **83:**6159–6163.
61. **Weiss, C. D., J. A. Levy, and J. M. White.** 1990. Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. J. Virol. **64:**5674–5677.
62. **White, J. M.** 1992. Membrane fusion. Science **258:**917–924.
63. **Wild, C., T. Oas, C. McDanal, D. Bolognesi, and T. Matthews.** 1992. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc. Natl. Acad. Sci. USA **89:**10537–10541.
64. **Wilk, T., T. Pfeiffer, and V. Bosch.** 1992. Retained *in vitro* infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. Virology **189:**167–177.
65. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85:**9580–9584.
66. **Willey, R. L., T. Klimkait, D. M. Frucht, J. S. Bonifacino, and M. A. Martin.** 1991. Mutations within the human immunodeficiency virus type 1 gp160

envelope glycoprotein alter its intracellular transport and processing. Virology **184:**319–329.
67. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3Å resolution. Nature (London) **289:**366–373.
68. **Yewdell, J. W., A. Yellen, and T. Bachi.** 1988. Monoclonal antibodies localize events in the folding, assembly, and intracellular transport of the influenza virus hemagglutinin glycoprotein. Cell **52:**843–852.
69. **Yu, X., X. Yuan, M. F. McLane, T.-H. Lee, and M. Essex.** 1993. Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions. J. Virol. **67:**213–221.