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Mutations in the Membrane-Spanning Domain of the Human Immunodeficiency Virus Envelope Glycoprotein That Affect Fusion Activity

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A chimeric protein consisting of the human immunodeficiency virus type 1 (HIV-1) envelope protein (Env) ectodomain joined to the transmembrane and cytoplasmic-tail domains of vesicular stomatitis virus G protein lost the ability to fuse CD4$^+$ HeLa cells yet was transported to the cell surface and cleaved normally. These results suggested some critical role of the HIV gp41 transmembrane or cytoplasmic domain in fusion. Subsequent mutagenic analysis of the HIV-1 Env transmembrane domain revealed that the sequence of amino acid residues from positions 696 to 707 of the transmembrane domain was important for fusion function but was not required for anchoring of the Env protein in the lipid bilayer or for transport to the cell surface. Further analysis indicated that the basic residues at positions 696 and 707 were critical for membrane fusion activity, as was the spacing between these residues. These results demonstrate that in addition to providing an anchoring function, the specific amino acid sequence in the transmembrane domain plays a crucial role in the membrane fusion process.

Two of the major functions of the human immunodeficiency virus (HIV) envelope protein are receptor binding and membrane fusion (13, 23). Like many viral fusion proteins, the HIV envelope protein undergoes a proteolytic activation step that is required for its fusion function (12). A cellular protease cleaves the gp160 precursor molecule into two subunits, gp120 and gp41, during transport to the plasma membrane (5, 27). Cleavage of the precursor reveals a sequence of hydrophobic amino acids at the N terminus of gp41 called the fusion peptide (24). Following receptor binding, it is generally believed that a conformational change occurs which facilitates an interaction between the fusion peptide and the receptor-bearing lipid bilayer, resulting in fusion between the host cell plasma membrane and the viral envelope (reviewed in references 2 and 14). Expression of the HIV envelope protein on the surface of CD4$^+$ cells also results in cell-cell fusion and formation of multinucleated syncytia (10). Beyond receptor binding, however, the molecular mechanism of HIV-induced membrane fusion is poorly understood. A previous study (8) showed that mutations involving some of the basic amino acid residues in and around the transmembrane (TM) domain of gp41 diminished gp120/41-induced cell fusion and reduced HIV infectivity. Many of these fusion-defective mutants were expressed efficiently on the cell surface, indicating that the mutations had no effect on transport. In a previous study (16), we constructed several hybrid proteins between the ectodomain of the HIV-1 envelope protein and the TM and cytoplasmic-tail domains of the vesicular stomatitis virus (VSV) G protein. Some of these constructs had fusion-defective phenotypes yet were also efficiently transported to the cell surface. To expand on the findings of Helseth et al. (8), we decided to examine the role that the membrane-spanning domain of the HIV Env protein plays in membrane fusion by analyzing the effect that additional mutations in this region have on syncytium formation in CD4$^+$ HeLa cell cultures.

Chimeric proteins between HIV Env and VSV G. In our previous studies, designed to determine whether the cytoplasmic domain of VSV G protein would affect incorporation of the HIV-1 envelope protein into VSV particles (16), we found that some VSV-G/HIV Env hybrid constructs had lost the ability to cause cell fusion in CD4$^+$ HeLa cultures (Fig. 1). One construct in which the VSV G protein TM and cytoplasmic domains replaced the corresponding domains of HIV Env (Env-G 665) and two others in which portions of the HIV Env TM domain were deleted (Env-G 685 and Env-G 696) were deficient in membrane fusion activity. In contrast, two other hybrid proteins in which the HIV Env TM domain was intact (Env-G 751 and Env-G 709) showed normal fusion. To determine whether the lack of fusion activity was a result of defective transport to the plasma membrane, we examined the levels of surface expression by using indirect immunofluorescence and flow cytometry (Fig. 1). The levels of surface expression for the two hybrid proteins causing fusion (Env-G 751 and Env-G 709) were similar to that of wild-type (wt) HIV Env. In addition, two of the three fusion-defective mutants, Env-G 696 and Env-G 665, were also expressed on the cell surface at levels equivalent to that of wt HIV Env. In contrast, Env-G 685 was not detected on the cell surface, which would explain the lack of fusion activity for this construct. Env-G 685 contains a stretch of only 17 hydrophobic and uncharged amino acids between Lys-665 and Lys-683 (TM sequence shown in Fig. 2). Presumably, this truncated TM domain interferes in some way with transport to the cell surface, perhaps through effects on protein folding. However, the fusion-defective phenotype of Env-G 696 and Env-G 665 could not be explained by a lack of transport.

A second possible explanation for the fusion-defective phenotype of the Env-G 696 and Env-G 665 hybrids could be lack of proteolytic cleavage during transport, because cleavage is required for membrane fusion activity (5, 12, 27). Radiolabeled

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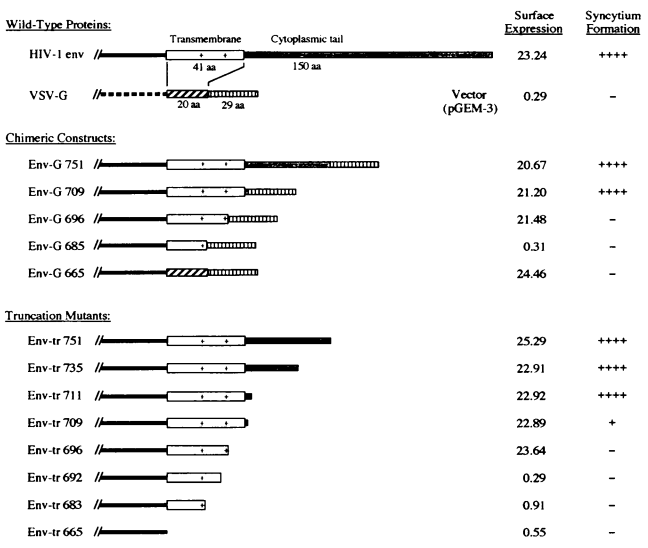

FIG. 1. Structures and functional characteristics of wt, chimeric, and truncated envelope proteins. A schematic view of each envelope protein is shown to compare the TM and cytoplasmic-tail domains and illustrate the regions involved in chimeric protein and truncation constructs. The numbers identifying each mutant represent the HIV-1 Env carboxy-terminal amino acid preceding either the VSV G cytoplasmic-tail sequence in chimeric proteins or a termination codon for truncation mutants. Plus symbols within the HIV Env TM domain represent basic amino acid residues Lys-683 and Arg-696 (see the TM sequence in Fig. 2). Plasmids encoding wt HIV-1 Env (pEnv-wt, BH10 strain), chimeric mutants Env-G 751 and Env-G 709, and truncation mutant Env-tr 735, under the control of the bacteriophage T7 promoter, have been described previously (16); construction of all other chimeric and truncation mutants employed the same methodology. HeLa cells were obtained from the American Type Culture Collection (Rockville, Md.), and CD4$^+$ HeLa (11) cells were from the AIDS Research and Reference Reagent Program. All cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (vTF7-3) was propagated in HeLa cells and purified as previously described (4). For quantitation of envelope proteins on the cell surface (surface expression), approximately 2×10^6 HeLa cells were infected with vTF7-3 at a multiplicity of infection of 10 and then transfected with 10 μg of Env plasmid DNA by using TransfectACE. The cells were incubated at 37°C for 12 h and then removed from the culture dish by incubation in 30 mM EDTA for 30 min at room temperature. After fixation with 3% paraformaldehyde, the cells were treated with sheep anti-gp120 serum (diluted 1/100) followed by fluorescein isothiocyanate-conjugated donkey anti-sheep immunoglobulin (diluted 1/100). Samples were washed three times with phosphate-buffered saline containing 10 mM glycine after fixation and after incubation (20 min at room temperature) with each antibody. An average of 5,000 cells were counted on a Coulter flow cytometer, and their fluorescence intensities were recorded. Surface expression values were determined as the product of mean fluorescence intensity times the percentage of fluorescing cells. For cell fusion experiments, approximately 10^6 CD4$^+$ HeLa cells were infected with vTF7-3 at a multiplicity of 10 and then transfected with 5 μg of plasmid DNA expressing Env proteins under the control of the T7 promoter by using TransfectACE (Life Sciences, Inc.). The cells were incubated for 12 to 24 h at 37°C and photographed by phase-contrast microscopy. Symbols represent qualitative estimates of the area involved in syncytium formation relative to the entire monolayer as follows: ++++, >50%; +, <5%; −, no syncytia observed. Quantitative measurements of syncytium formation in monolayer cultures are dependent on counting of the average number of nuclei present in polykaryons; however, when >50% of the monolayer is fused, these counts are inaccurate because the nuclei are piled on top of one another. Use of this method to determine quantitative levels of syncytium formation required examination of the cultures at earlier time points (10 h, when 10 to 100 nuclei were present in polykaryons); thus, mutants that exhibited low levels (+) of fusion at 24 h were scored as completely negative at 10 h (data not shown). The cytopathic effects of vaccinia virus infection prevented analysis of HIV Env-induced fusion beyond 24 h postinfection.

Env proteins from cell lysates and media were analyzed by radioimmunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 3A). We found that each chimeric protein was expressed in cells at a level comparable to that of the wt HIV-1 Env protein. The small differences in the mobilities of the proteins precipitated from cell lysates were consistent with their predicted molecular weights. The proteolytic subunits gp120 and gp41 were poorly resolved under these conditions, presumably because only a small fraction of the total envelope protein expressed by cells is processed (27). Because of the loose association between gp120 and gp41, gp120 is readily released into the culture medium (17, 21). The presence of gp120 in the culture medium is therefore an indicator of transport and processing. The levels of gp120 in the medium of cells expressing fusion-positive mutants Env-G 751 and Env-G 709 and in that of cells expressing fusion-deficient mutants Env-G 696 and Env-G 665 were equivalent to that in the medium of cells expressing the wt Env, indicating normal proteolytic processing (Fig. 3A). In contrast, no gp120 was observed in the medium of cells expressing Env-G 685, the hybrid which was not expressed on the cell surface. Because processing of the HIV-1 Env precursor (gp160) occurs late during transport (27), these results suggested that transport of Env-G 685 was blocked at an early stage. When we examined these cells by indirect intracellular immunofluorescence microscopy, the Env-G 685 mutant exhibited a perinuclear staining pattern consistent with localization to the endoplasmic reticulum (ER), while all other mutants exhibited a diffuse staining pattern similar to that of wt HIV-1 Env (data not shown). These results, together with the flow cytometry data (Fig. 1), suggest that the fusion-defective phenotype of Env-G 685 is due to a block in transport from the ER; however, some other mechanism must be responsible for the failure of Env-G 696 and Env-G 665 to cause fusion. Because the loss in fusion activity in mutant Env-G 696 was associated with the deletion of amino acids (aa) 697 to 709 in the HIV-1 Env TM domain, we carried out a more detailed analysis of sequence changes in this region and their effects on membrane fusion activity.

Truncation mutants. Several truncation mutations were made by introducing translation termination codons into the HIV-1 Env gene (Fig. 1). These mutations ranged from deletion of 105 carboxy-terminal aa from the 150-aa cytoplasmic tail (Env-tr 751) to deletion of the entire membrane-spanning and cytoplasmic-tail domains (Env-tr 665). We examined the expression of each truncation mutant by radioimmunoprecipitation and SDS-PAGE and found that all of the mutant proteins were expressed intracellularly at levels similar to that of wt Env (Fig. 3B). In addition, mutants Env-tr 751, Env-tr 735, Env-tr 711, Env-tr 709, and Env-tr 696 were expressed on the cell surface at levels equivalent to that of the wt Env protein (Fig. 1). Furthermore, these truncation mutants all released gp120 into the culture medium at wt levels (Fig. 3B). In contrast, mutant Env-tr 692 appeared to have a defect in transport because gp120 release into the medium was not seen (Fig. 3B) and the protein was not detected on the cell surface (Fig. 1). Examination of Env-tr 692 by intracellular immunofluorescence revealed a perinuclear staining pattern

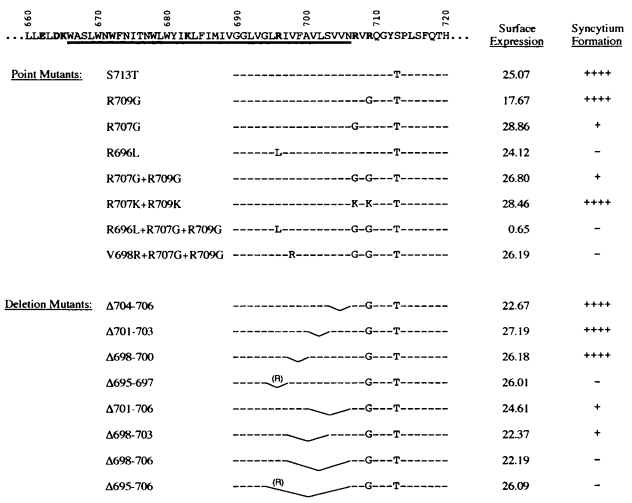

FIG. 2. Structures and functional characteristics of point and deletion mutants. The sequence of HIV Env amino acids from residues 660 to 720, encompassing the TM domain (underlined), is shown at the top (one-letter symbols) to identify the residues involved in point mutations and deletions. The name of each point mutant identifies (i) the original wt amino acid (first letter), (ii) its position in the HIV Env amino acid sequence (number), and (iii) the amino acid to which the original was mutated (last letter). Dashes represent residues that have not been changed. All point mutants contain the S713T change, and all deletion mutants contain both the S713T and R709G changes, even though their names do not indicate this. To simplify the production of point and deletion mutations within the TM domain, we first generated an HIV-1 Env plasmid that contained a convenient restriction site near the junction of the TM and cytoplasmic-tail domains. Point mutant S713T was made by PCR amplification (18) with a primer (5′-GTCC CCTCGGGATTGGGAGGTGGGTCTGAAACGATAATGG**TGTA** TACCCCTGCCTAA-3′) that encoded a new *Acc*I restriction site (underlined) and a corresponding codon change from Ser-713 to Thr (bold, antisense) and overlapped the *Ava*I restriction site (double underlined) in the cytoplasmic-tail coding region of HIV-1 Env. The second primer overlapped the *Hin*dIII restriction site in the HIV-1 Env ectodomain coding region and has been described previously (16). This PCR product, along with the cytoplasmic-tail coding fragment from the *Ava*I site to a *Hin*dIII site in the polylinker sequence of the plasmid vector, was ligated to pEnv-wt, which had been digested with *Hin*dIII. All other point and deletion mutants were generated in a similar way, except that the mutagenic oligonucleotide primers overlapped the new *Acc*I restriction site at codon 713 and the cytoplasmic-tail coding fragment extended from the *Acc*I site to the *Hin*dIII site in the polylinker sequence. The DNA sequences of all regions generated by PCR were confirmed by dideoxynucleotide sequencing (19) with Sequenase (U.S. Biochemical Corp.). Surface expression and cell fusion levels were analyzed as described in the legend to Fig. 1.

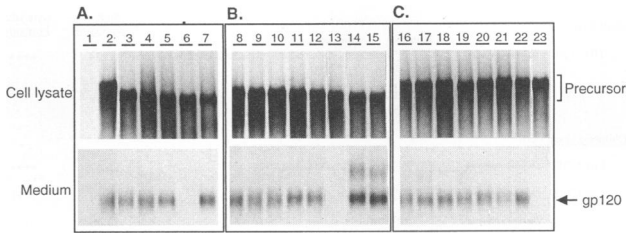

FIG. 3. Expression and processing of envelope proteins. (A) Chimeric constructs. Lanes: 1, vector pGEM3; 2, wt HIV-1 Env; 3, Env-G 751; 4, Env-G 709; 5, Env-G 696; 6, Env-G 685; 7, Env-G 665. (B) Truncation constructs. Lanes: 8, Env-tr 751; 9, Env-tr 735; 10, Env-tr 711; 11, Env-tr 709; 12, Env-tr 696; 13, Env-tr 692; 14, Env-tr 683; 15, Env-tr 665. (C) Point mutants. Lanes: 16, S713T; 17, R709G; 18, R707G; 19, R696L; 20, R707G+R709G; 21, R707K+R709K; 22, V698R+R707G+R709G; 23, R696L+R707G+R709G. Approximately 10^6 HeLa cells were infected with vTF7-3 at a multiplicity of 10 and transfected with 5 μg of Env plasmid DNA by using Transfect ACE. At 8 h posttransfection, the cells were incubated for 15 min in methionine-free medium, 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 centrifuged at 10,000 × *g* for 2 min to remove cell debris. SDS was added to 0.2% in the cell lysates, and the medium samples were adjusted to 1% Nonidet P-40 and 0.2% SDS. Samples were incubated at 4°C for 18 h in the presence of human anti-HIV-1 sera, and immune complexes were precipitated with excess fixed *Staphylococcus aureus* (Pansorbin; Calbiochem). The samples were analyzed by SDS–10% PAGE and autoradiography.

consistent with ER localization (data not shown). The more severely truncated mutants Env-tr 683 and Env-tr 665, lacking half or all of the TM domain, respectively, were also not found on the cell surface (Fig. 1) and were apparently secreted because uncleaved precursor proteins of the expected size were found in the culture medium (Fig. 3B). Env-tr 683 and Env-tr 692 differed with respect to transport, even though both proteins contained the same sequence of 17 hydrophobic and uncharged residues between Lys-665 and Lys-683. While Env-tr 692 appeared to be blocked in transport from the ER, as was the chimeric construct Env-G 685, Env-tr 683 was efficiently secreted into the culture medium. Therefore, truncation after Lys-683 results in a protein that is not stably anchored in the membrane but is still transported and proteolytically processed, whereas the presence of nine additional amino acids (Env-tr 692) or the cytoplasmic tail of VSV G (Env-G 685) results in proteins that may be anchored but not transported.

Next we determined whether the truncations had an effect on the membrane fusion activity of the mutant proteins by expressing them in CD4$^+$ HeLa cells (Fig. 1). Truncation of the envelope protein to aa 711 (Env-tr 711) had no effect on syncytium formation, whereas truncation of two more aa (Env-tr 709) and beyond (Env-tr 696 to Env-tr 665) greatly reduced or eliminated fusion activity. In a comparison of syncytium formation and cell surface expression, we found two mutants that were expressed at normal levels on the cell surface yet were deficient in fusion, i.e., Env-tr 709 and Env-tr 696. These mutants, like the chimeric proteins, suggested that some important sequence affecting fusion was located at aa 697 to 709. On the basis of these results, we concentrated on generating more subtle site-specific mutations in the region of aa 696 to 709 in the TM domain.

Point mutants. Comparative analysis of all of the HIV-1, HIV-2, and simian immunodeficiency virus protein sequences in the SwissProt data bank revealed that charged amino acids Arg-696, Arg-707, and Arg-709 (HIV-1 Env [BH10 strain] numbering) are highly conserved, as is the spacing between them. Therefore, we decided to examine the effects of changing these basic amino acids to uncharged residues and to alter the distance between aa 696 and aa 707 by introducing deletions. To simplify mutagenesis in this region, we first generated a mutation which created a convenient restriction endonuclease site in close proximity to the TM domain. A search of all possible restriction sequences unique to the Env plasmid did not reveal a site which could be generated without a corresponding codon change; therefore, we opted to make a conservative change of serine to threonine at position 713 to

generate an *Acc*I site. When the transport and fusion properties of this mutant (S713T) were compared to those of wt Env (Fig. 2), no differences were found. Thus, mutant S713T was used as the parent plasmid for all subsequent mutagenesis. Figure 2 illustrates the various point mutations that were generated.

Expression and transport of the point mutants were analyzed by radioimmunoprecipitation and SDS-PAGE and by immunofluorescence and flow cytometry. In SDS-PAGE analysis (Fig. 3C), we found that all point mutant proteins were expressed intracellularly at levels similar to that of wt Env and that gp120 was released into the culture medium from all point mutant-expressing cells except one, R696L+R707G+R709G, the mutant in which all three basic residues were changed. Immunofluorescence and flow cytometry demonstrated that all point mutants, except for R696L+R707G+R709G, were expressed efficiently on the cell surface (Fig. 2). These data suggested that mutant R696L+R707G+R709G was defective in transport to the cell surface. We examined the point mutants by intracellular immunofluorescence microscopy and found that mutant R696L+R707G+R709G was localized to the perinuclear region, indicative of a block in transport at the ER, while all of the other mutants exhibited a diffuse staining pattern similar to that of wt Env (data not shown). Therefore, changing all three Arg residues (696, 707, and 709) to uncharged amino acids resulted in a transport-defective protein, whereas all single and double mutants and one triple mutant (V698R+R707G+R709G) retained transport activity. These data, together with the data from Env-G 685 and Env-tr 692, suggest that at least one charged amino acid is required in the region encompassing residues 696 to 709 for transport to the cell surface.

Next we examined the fusion characteristics of the point mutants by expressing them in $CD4^+$ HeLa cells (Fig. 2). Changing the Arg at position 709 to Gly (R709G) had no effect on fusion activity. In contrast, a change of Arg to Gly at position 707 (R707G) reduced fusion activity to barely detectable levels, and a change of Arg to Leu at position 696 (R696L) eliminated fusion activity altogether. The double mutant R707G+R709G behaved like the single mutant R707G in that syncytium formation was observed only rarely. On the other hand, changing both of these Arg residues to Lys (R707K+R709K) had no effect on fusion, indicating that Arg is not stringently required at position 707. Finally, we used the double mutant R707G+R709G, which demonstrated minimal fusion activity, to introduce a third mutation of Val to Arg at position 698 (V698R+R707G+R709G). The rationale behind this mutation was that it might define a new TM–cytoplasmic-tail boundary at positions 696 to 698 and restore fusion activity. This mutant lost all fusion activity, although it was transported to the cell surface efficiently (Fig. 2).

Deletion mutants. Next we decided to examine the role that the 10 hydrophobic or uncharged aa residues between Arg-696 and Arg-707 play in membrane fusion. The rationale behind this series of mutations was to determine whether specific amino acids were required within this sequence or whether the spacing between Arg-696 and Arg-707 was important. We were also interested in defining the minimum amino acid sequence requirement for retention of full fusion activity. Since Arg-709 appeared to be dispensable for fusion, we decided to use mutant R709G as the parent for generation of additional mutations. Several deletions, ranging from 3 to 12 aa, were generated between residues 694 and 707, and their effects on transport and cell fusion were determined (Fig. 2). Three mutants in which different sets of three amino acids were deleted (Δ704-706, Δ701-703, and Δ698-700) exhibited normal fusion activity. However, when the three amino acids from Leu-695 to Ile-697 were deleted (Δ695-697), fusion activity was lost, presumably because of the loss of Arg-696. Deletions of 6 aa at two different positions (Δ701-706 and Δ698-703) greatly reduced the level of syncytium formation, and deletions of 9 and 12 aa (Δ698-706 and Δ695-706, respectively) abolished fusion entirely. In all of the deletion mutants, however, expression on the cell surface was equivalent to that of wt Env (Fig. 2), indicating that the sequence between residues 694 and 707 is not required for membrane anchorage or transport. These results also demonstrate that no particular residues between aa 696 and 707 are essential for syncytium formation, yet the distance (or perhaps hydrophobicity) between residues 696 and 707 is sensitive to change.

In summary, we have shown that approximately 30% of the carboxy-terminal portion of the HIV-1 Env TM domain is not needed for membrane anchoring or transport to the cell surface yet is crucial for the membrane fusion function of the protein. Some chimeric proteins in which all or part of the Env TM and cytoplasmic-tail domains had been replaced by analogous regions from the VSV G protein had lost the ability to cause cell fusion but were still transported to the cell surface efficiently. In a related study (26), it was found that a hybrid protein consisting of the extracellular domain of rabies virus G protein linked to the TM and cytoplasmic domains of VSV G had also lost fusion activity, yet this hybrid protein trimerized and was transported to the cell surface efficiently. Further analysis of HIV Env through the use of truncation mutations confirmed that the sequence of amino acids making up the carboxy-terminal end of the TM domain was required for fusion but not transport or anchoring. This sequence is punctuated by Arg-696, which lies within the TM domain as defined by hydropathy analysis, and Arg-707, which forms the junction between the TM and cytoplasmic-tail domains. These two residues are particularly sensitive to change.

In a previous study by Helseth et al. (8), the role of Lys-683, Arg-696, Arg-707, and Arg-709 in membrane fusion and virus infectivity was examined. They found that a mutation of Lys-683 to Ile resulted in a 95% reduction in syncytium formation in a cocultivation assay. In addition, a mutation which replaced Arg-696 with the four-amino-acid sequence VGLS was also deficient in cell fusion (86% reduction). In contrast, replacement of Arg-696 with Ser led to an only 13% reduction in syncytium formation (8). In the present study, however, we found that every mutation involving Arg-696 resulted in complete loss of cell fusion. Similarly, when we mutated Arg-707 to Gly, syncytium formation was reduced by more than 90%, whereas the mutation of Arg-707 to Ile in the study by Helseth et al. resulted in only 35% reduction. One possible explanation for the apparent differences between our results and those of Helseth et al. may lie in the different amino acids which were substituted. Perhaps exchanging the basic residue at position 696 with a hydrophobic Leu residue has more of an effect on fusion than does mutating it to a polar Ser residue. Similarly, a nonpolar Gly residue at position 707 may be more detrimental to fusion than a hydrophobic Ile residue. We employed a different fusion assay, which also may have contributed to the different results. It is important to note, however, that our studies are in agreement with the findings by Helseth et al. that mutations at positions 696 and 707 that affect cell fusion do not disrupt membrane anchoring or cell surface transport.

It is generally believed that the function of the fusion peptide at the amino terminus of gp41 (and of other viral fusion proteins) is to interact with and destabilize target membranes (3, 6, 7, 22). However, it is clear that for membrane

fusion to occur, two lipid bilayers must be disrupted. Two models have been proposed that suggest that the fusion peptide of influenza virus hemagglutinin destabilizes both the target membrane and hemagglutinin-bearing membrane (reviewed in reference 25). Both of these models are based on the low-pH-induced conformational change in hemagglutinin that occurs preceding fusion. Because HIV-mediated fusion occurs at neutral pH (23), its mechanism of fusion is probably quite different from that of hemagglutinin, and the structural differences between the membrane-spanning domains of these two proteins may reflect this mechanistic distinction. A conformational change in the gp120/41 complex is triggered by binding to CD4 (1, 9, 15, 20). Conceivably, the mutations in the TM domain affect the ability of the protein to undergo this change. Another possibility is that the subdomain encompassing residues 696 to 707 plays some direct role in destabilizing the Env-bearing lipid bilayer following receptor binding to allow lipid redistribution between the target membrane and viral envelope. The effects of these and additional mutations in the membrane-spanning domain on oligomerization, CD4 binding, and virus infectivity are under investigation.

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