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Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41

(retrovirus/transmembrane/AIDS/syncytium)

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ABSTRACT **The human immunodeficiency virus transmembrane glycoprotein gp41 has at its amino terminus a strongly hydrophobic stretch of 28 amino acids flanked by a highly conserved series of polar amino acids. To investigate the role in syncytium formation of the hydrophobic amino terminus of gp41 and the polar border of this hydrophobic region, we introduced eight single-amino acid substitutions and one double-amino acid substitution in the amino-terminal 31 amino acids of gp41. The mutant envelope glycoproteins were expressed from two distinct human immunodeficiency virus type 1 envelope glycoprotein expression vectors; the effects of the mutations on syncytium formation, envelope glycoprotein transport, secretion, and CD4 receptor-binding were analyzed. Results showed that polar substitutions throughout the hydrophobic amino terminus of gp41 greatly reduced or blocked syncytium formation mediated by the human immunodeficiency virus type 1 envelope glycoproteins, as did nonconservative mutations in the polar border of the hydrophobic amino terminus. Mutations at gp41 amino acids 15, 26, and 29 also significantly increased the extent of gp120 secretion into the extracellular medium. None of the mutations detectably affected envelope glycoprotein processing or envelope glycoprotein binding to CD4.**

Enveloped viruses, under appropriate conditions, induce cell fusion, or syncytium formation, upon infection of permissive cell types. Early descriptions of such virus-induced cell fusion involved the orthomyxoviruses and paramyxoviruses (1–3), and one of the early retroviral plaque assays depended upon the ability of murine leukemia viruses to induce syncytium formation in the XC cell line (4). Later work indicated that expression of the orthomyxovirus envelope proteins was necessary and sufficient to induce cell fusion (5). More recently, syncytium formation has been described as a characteristic of human immunodeficiency virus (HIV) infection of $CD4^+$ cells, and, as demonstrated for the orthomyxoviruses and paramyxoviruses (3), the envelope glycoproteins have been shown to be responsible for this cell-fusion property (6, 7). The syncytium formation induced by HIV leads to cell death in culture and may contribute to the depletion of $CD4^+$ helper T cells in AIDS patients (6–10). Membrane fusion is also an essential step in the infection process (11).

HIV envelope glycoproteins, like those of other retroviruses, are synthesized as a polyprotein precursor that is cleaved to generate the surface and transmembrane (TM) envelope proteins (12, 13). These two mature proteins remain associated after cleavage of the precursor molecule. The surface protein contains the receptor-binding domains, while the TM protein anchors the surface–TM protein complex in the viral envelope or host-cell membrane. For HIV, the surface and TM envelope glycoproteins and the envelope precursor have been termed gp120, gp41, and gp160, respectively.

The overall organization of retroviral envelope proteins bears a striking similarity to the envelope proteins of other enveloped viruses—most notably those of the orthomyxoviruses and paramyxoviruses. The TM envelope proteins of these viruses generally contain at their amino termini a region composed almost entirely of hydrophobic amino acids (AAs). Studies conducted over the last decade have implicated this hydrophobic region in viral membrane fusion functions. Carbobenzoxy-derivatized peptides with sequences homologous to regions within the hydrophobic amino termini of the orthomyxovirus and paramyxovirus TM envelope proteins block viral infectivity and fusion activity (14, 15). Single-AA changes within the hydrophobic amino terminus of the influenza hemagglutinin protein HA_2 blocked fusion (16), and peptides corresponding to the amino terminus of influenza protein HA_2 were shown to be capable of inducing membrane fusion (17, 18). Because of their role in fusion, the hydrophobic amino-terminal regions of the orthomyxovirus and paramyxovirus TM proteins were termed "fusion peptides." Several studies suggested that the analogous hydrophobic regions at the amino termini of retroviral TM envelope proteins were also involved in the cell-fusion properties of these proteins. Linker insertion mutations introduced after AA 10 of the HIV-1 gp41 glycoprotein blocked cell fusion (19), as did point mutations at positions 8 and 9 of gp41 (20). Mutagenesis of the TM glycoprotein of the related simian immunodeficiency virus also suggested a role for this hydrophobic region in cell fusion (21).

Although the hydrophobic amino-terminal regions of the HIV and simian immunodeficiency virus TM envelope glycoproteins extend for nearly 30 AAs, to date no single AA mutations have been introduced beyond AA 9 of the HIV-1 TM glycoprotein gp41 or beyond AA 10 of the simian immunodeficiency virus TM glycoprotein (20, 21). We were interested in determining the extent of the hydrophobic amino-terminal region involved in cell fusion. We also noted that a highly conserved series of polar AAs was located at the end of the hydrophobic stretch (22–28) and speculated that this polar border might also be involved in cell fusion. To examine these questions, we introduced point mutations throughout the entire length of the amino-terminal hydrophobic stretch of the HIV-1 TM glycoprotein gp41 and in the highly conserved polar border of this hydrophobic sequence. We analyzed the effects of these mutations on cell fusion and envelope glycoprotein processing, transport, and association and on envelope glycoprotein binding to the CD4 receptor molecule. These studies were conducted by using two distinct HIV-1 envelope glycoprotein expression vectors, one of which expressed high levels of envelope glycoproteins and the other of which expressed lower levels of envelope gly-

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Abbreviations: AA, amino acid; HIV, human immunodeficiency virus; TM, transmembrane.

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coproteins. This technique enabled us to examine the effects of the gp41 mutations in the absence of viral replication and under conditions of varied envelope glycoprotein expression.

MATERIALS AND METHODS

Cells and DNA. The $CD4^+$ HeLa cell line HeLa T4 (29) was provided by R. Axel (Columbia University). Plasmids pIIIenv3-1 (7) and pNL4-3 (30) were provided by J. Sodroski (Harvard University) and M. Martin (National Institute of Allergy and Infectious Diseases), respectively.

DNA Mutagenesis and Cloning. Point mutations were introduced with 17-base oligonucleotides synthesized by the University of Wisconsin Biotechnology Center or by Genetic Designs (Houston). The template for mutagenesis was an M13 subclone constructed using the *Kpn* I and *Hin*dIII sites located in the *env* gene. All mutations, except for 41.29 and 41.1B/8, were introduced into a template derived from the pIIIenv3.1 *env* subclone, which was constructed from the BH10 proviral clone (7). The 41.29 and 41.1B/8 mutants were introduced into a template derived from pNL4-3 (30). The envelope glycoproteins expressed from these two proviral clones induce syncytium formation with comparable efficiencies (data not shown). Mutagenesis was performed by the gapped duplex method (31) by using the $dUTPase^-$,UNG^- strain of *Escherichia coli* RZ1032 (32). Hybridizing and extension reactions were done as described (33). After mutagenesis reactions, the 520-base-pair (bp) *Bgl* II–*Hin*dIII fragment was sequenced in its entirety and reintroduced into the full-length *env* gene. Cloning was accomplished by standard procedures (34), and DNA sequencng was done with a Sequenase kit (United States Biochemical).

Expression of HIV-1 Envelope Glycoproteins. The vectors that we constructed for the expression of HIV-1 envelope glycoproteins have been described (35). pNL4-3dPst is a *gag/pol* deletion construct of the full-length HIV-1 proviral clone pNL4-3 (30) made by deleting the region from the *Pst* I site in *gag* to the *Pst* I site in *pol*. We constructed pHenv by cloning the *Sal* I–*Bam*HI region of pNL4-3 (containing the *env*, *tat*, and *rev* open reading frames) immediately adjacent to the part of the HIV-1 long terminal repeat provided by pIIIenv3-1 (7). pNL4-3dPst and pHenv induce ≈200 and 800 syncytia per μg of DNA, respectively, when transfected into the $CD4^+$ HeLa cell line HeLa T4 (35). The HIV-1 envelope glycoprotein expression vectors pNL4-3dPst and pHenv are currently available through the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases).

Cell Culture and Fusion Assays. The HeLa T4 cell line used in these studies (29) was maintained at 37°C in Dulbecco's modified Eagle's medium containing 5% calf serum (HyClone) and G418 at 900 μg/ml (GIBCO). G418 was added during cell passaging to ensure maintenance of the plasmid expressing CD4 (29). One day before transfection with 10–15 μg of plasmid DNA, cells were seeded at 0.8×10^6 per 60-mm gridded dish and cultured as above without G418. Transfections were done by the calcium phosphate procedure as described (36). Two to 3 days posttransfection cells were stained with a solution of 5 g of methylene blue per liter and 1.7 g of pararosamiline per liter in methanol and were scored for syncytia. Syncytia were scored microscopically by counting the giant cells (containing greater than four nuclei) on the gridded transfection plates. Equal numbers of grids were scored on plates transfected with wild-type or mutant envelope glycoprotein expression vectors. Syncytia induced by transfection with our envelope expression vectors were morphologically indistinguishable from those induced by infection with HIV-1 (35).

Cell Labeling, Radioimmunoprecipitation, and Immunofluorescence. Methods used in cell labeling, preparation of cell lysates, and radioimmunoprecipitations have been described (35, 36). Anti-envelope immunoprecipitations were performed with AIDS patient sera provided by R. Tomar (University of Wisconsin, Madison). Indirect immunofluorescence was performed as described (37). AIDS patient sera was the source of anti-envelope glycoprotein antibodies; goat anti-human IgG conjugated with fluorescein isothiocyanate was used as the secondary antibody.

CD4 Binding Assays. Transfected, envelope glycoprotein-expressing HeLa T4 cells were labeled for 15 hr with [^{35}S]methionine. Lysates were then prepared and immunoprecipitated in parallel with either OKT4, an anti-CD4 mouse monoclonal antibody that can bind CD4 complexed with HIV envelope glycoproteins or OKT4A, which cannot bind CD4 complexed with HIV envelope glycoproteins (38). These anti-CD4 immunoprecipitates were then precipitated with AIDS patient sera containing high titers of anti-envelope glycoprotein antibodies. The precipitates from this second immunoprecipitation were then subjected to SDS/PAGE and analyzed by fluorography as described (36). Envelope glycoproteins were precipitated in the first (anti-CD4) immunoprecipitation by their association with CD4. Precipitation of envelope–CD4 complexes by OKT4 but not OKT4A showed the specificity of binding between CD4 and HIV envelope glycoproteins. The second (anti-envelope) immunoprecipitation was done to reduce background. OKT4 and OKT4A were purchased from Ortho Diagnostic.

RESULTS

Mutagenesis of the Hydrophobic Amino Terminus of gp41. Using oligonucleotide-directed mutagenesis, we introduced mutations into the hydrophobic amino-terminal end of gp41 and into the highly conserved polar border of this hydrophobic stretch. The changes introduced into the hydrophobic region (Fig. 1) were as follows: AA 1, Ala → Glu; AA 2, Val → Glu; AA 9, Leu → Arg; AA 15, Ala → Glu; AA 26, Leu → Arg; and AA 28, Val → Glu. A double mutation was also introduced: AA 1, Ala → Val and AA 8, Phe → Leu. In the polar border of the hydrophobic region the following changes were made: AA 29, Gln → Leu and AA 31, Arg → Gly. After mutagenesis reactions, sequencing was done to confirm the introduced mutations and to determine whether or not mutations at sites other than those specified by the oligonucleotides had arisen during mutagenesis. Only the desired mutations were found to have been introduced. The mutated regions were then cloned into the envelope glycoprotein expression vectors pNL4-3dPst and pHenv (see *Materials and Methods*).

Effects of gp41 Mutations on Syncytium Formation. To determine the effects of the gp41 mutations on syncytium formation, the envelope glycoprotein expression vectors pNL4-3dPst and pHenv containing the mutations were introduced by transfection into the $CD4^+$ HeLa cell line HeLa T4. Two days after transfection, cells were stained and scored for syncytia. The results of these studies are presented in Table 1. The Ala → Glu change at gp41 AA 1 greatly reduced but did not completely block syncytium formation. Polar substitutions at gp41 AAs 2, 9, 15, and 26 completely abolished syncytium formation. A Val → Glu substitution at the last residue of the hydrophobic amino-terminal region (AA 28) greatly reduced but did not completely abolish syncytium formation. The double conservative mutation (positions 1 and 8) reduced the efficiency of syncytium formation. In the polar border of the hydrophobic amino-terminal region, the AA 29 Gln → Leu mutation completely abrogated cell fusion, whereas the AA 31 Arg → Gly mutation reduced but did not abolish syncytium formation. As determined by immunofluorescence, these mutations did not sig-

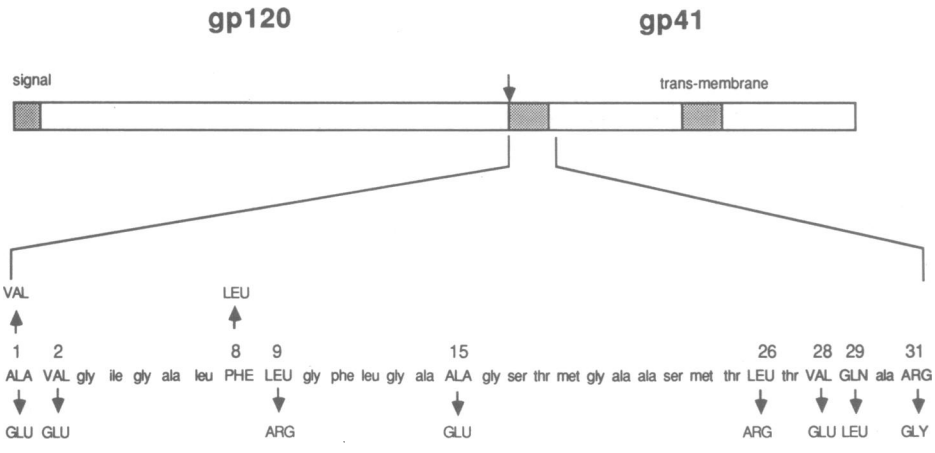

FIG. 1. Mutations introduced into the amino-terminal region of gp41. Wild-type AA sequence is shown, and numbers indicate the mutated positions. Arrows above sequence designate the double AA mutation (41.1B/8); arrows below sequence designate single AA mutations. Major hydrophobic regions of the HIV envelope glycoprotein are shown as shaded regions; signal peptide and transmembrane domain are labeled. The arrow between gp120 and gp41 indicates the gp160 cleavage site.

nificantly alter the number of transfected cells expressing envelope glycoproteins (data not shown).

Effects of gp41 Mutations on Envelope Glycoprotein Expression and Processing. Uncleaved retroviral envelope glycoprotein precursors are not competent to induce syncytium formation (35, 36, 39). To determine whether a defect in envelope precursor cleavage was playing a role in the fusion-negative phenotype of the gp41 mutants, we examined by radioimmunoprecipitation the cell-associated expression of the mutant envelope glycoproteins. Two days after transfection with wild-type or mutant pHenv constructs, HeLa T4 cells were labeled with [^{35}S]methionine for 15 hr, lysed, and immunoprecipitated with AIDS patient serum containing high titers of anti-envelope glycoprotein antibodies. A representative immunoprecipitation of cell-associated envelope glycoproteins is presented in Fig. 2*A*. The gp160 and gp120 bands were excised from this and repeat gels, and radioactivity was counted by liquid scintillation. Cell-associated gp160/gp120 ratios, calculated from the averages of at least three assays, were 1.0, 1.0, 1.1, 1.4, 2.6, 3.5, 1.7, 2.3, 0.8, and 1.5 for wild-type, mutant 41.1, mutant 41.2, mutant 41.9, mutant 41.15, mutant 41.26, mutant 41.28, mutant 41.29, mutant 41.31, and mutant 41.1B/8, respectively. The results of this analysis indicated that the 41.1, 41.2, 41.9, 41.28, 41.1B/8, and 41.31 mutations (Fig. 2*A*, lanes 3, 4, 5, 7, 8, and 12, respectively) did not significantly affect the ratio of cell-associated gp160 to gp120, whereas the 41.26, 41.15, and, to a lesser extent, the 41.29 mutations (Fig. 2*A*, lanes 6, 10, and 11, respectively) increased the cell-associated gp160/gp120 ratio. A reproducible reduction in the amount of gp41 found in envelope glycoprotein-expessing cells also resulted from the 41.15 and 41.29 mutations (Fig. 2*A*, lanes 10 and 11, respectively).

Table 1. Effects of gp41 mutations on syncytium formation

Mutation*	Change	Relative syncytium formation	
		pNL4-3dPst	pHenv
Wild type	—	100	100
gp41.1	Ala → Glu	4	8
gp41.2	Val → Glu	<2	<2
gp41.9	Leu → Arg	<2	<2
gp41.15	Ala → Glu	<2	<2
gp41.26	Leu → Arg	<2	<2
gp41.28	Val → Glu	<2	23
gp41.29	Gln → Leu	<2	<2
gp41.31	Arg → Gly	11	68
gp41.1B/8	Ala → Val Phe → Leu	4	56

All data represent averages of at least six assays.
*Number after period indicates AA position mutated.

The surface glycoprotein gp120 is efficiently shed from the surface of cells expressing the HIV envelope glycoproteins (40). As a measure of envelope glycoprotein transport to the cell surface and the association between gp120 and gp41, the extent of gp120 release from expressing cells was determined by collecting the medium from labeled cells, removing unattached cells by low-speed centrifugation, and immunoprecipitating with AIDS patient serum. An immunoprecipitation of secreted gp120 is presented in Fig. 2*B*. To quantify the relative amounts of intracellular vs. extracellular gp120, the gp120 bands from these gels were excised, and radioactivity was counted by liquid scintillation and compared with values obtained from counting the intracellular gp120 in the assays discussed above. The 41.1, 41.2, 41.9, 41.28, 41.1B/8, and 41.31 mutations (Fig. 2*B*, lanes 3, 4, 5, 7, 8, and 12, respectively) did not significantly affect the extent of gp120 secretion. Band counting indicated that the 41.26 and 41.15 mutations resulted in at least a 3-fold increase in the ratio of extracellular to cell-associated gp120. Although the increase in extracellular gp120 was not as dramatic with the 41.29 mutant, comparison of the extracellular and cell-associated gp120 in lanes 9 of Fig. 2 *A* and *B* (wild type) and lanes 11 of Fig. 2 *A* and *B* (mutant 41.29) demonstrates that the 41.29 mutation increased the relative amount of extracellular gp120. That significant amounts of cell-associated or secreted gp120 were expressed by all mutants indicated that none of the mutations blocked cleavage of gp160 to gp120 and gp41.

These results suggested that none of the gp41 mutations impaired transport of the mutant envelope glycoproteins to the cell membrane. Defects in transport to the cell surface would have been apparent by dramatic reductions in the amount of gp120 detected in the cell medium. Furthermore, because the HIV envelope glycoproteins are heavily glycosylated and because the larger, complex oligosaccharide side chains are added in the Golgi body during transport to the cell membrane, any defects in transport would be expected to yield significant differences in the sizes of mutant envelope glycoproteins; such size differences are not apparent (Fig. 2).

To further investigate the extent to which mutant envelope glycoproteins were transported to the cell surface, we performed cell-surface immunofluorescence by using AIDS patient sera specific for gp41. In two to four experiments with each mutant, the amount of gp41 detected on the surface of cells transfected with mutant envelope glycoprotein expression constructs was found comparable to the amount expressed on cells transfected with wild-type constructs (data not shown).

Effect of gp41 Mutations on CD4 Binding. Binding of gp120 to the receptor molecule CD4 is thought to be a prerequisite to the fusion process. It was therefore necessary to determine whether or not the gp41 mutations affected binding of the envelope glycoproteins to CD4. Our CD4-binding assay is

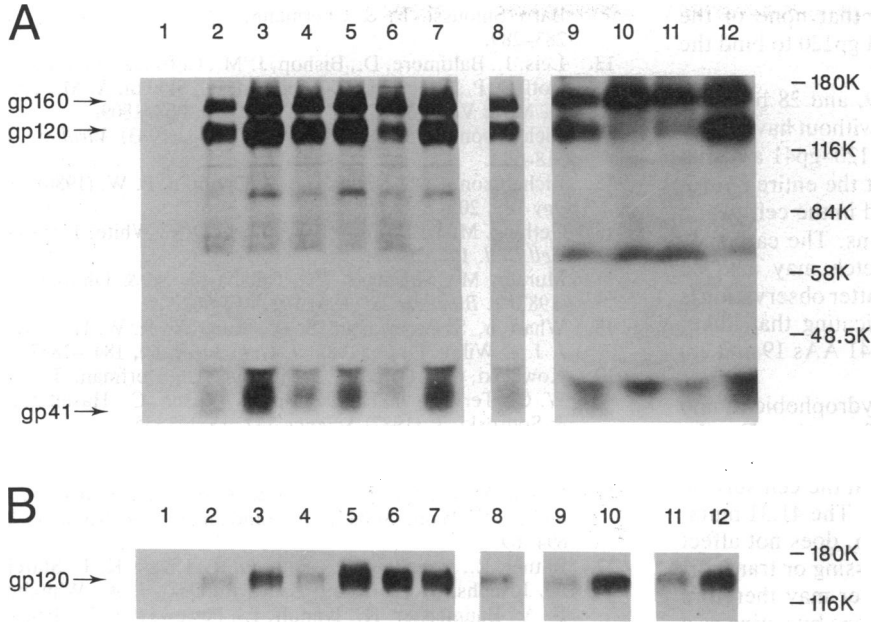

FIG. 2. Immunoprecipitation of intracellular and secreted HIV-1 envelope glycoproteins. (*A*) Intracellular glycoproteins. (*B*) Secreted glycoproteins. Lanes: 1, untransfected HeLa T4; 2, wild type; 3, mutant 41.1; 4, mutant 41.2; 5, mutant 41.9; 6, mutant 41.26; 7, mutant 41.28; 8, mutant 41.1B/8; 9, wild type; 10, mutant 41.15; 11, mutant 41.29; 12, mutant 41.31. Positions of gp160, gp120, and gp41 are indicated at left; positions of molecular size markers in kDa (K) are indicated at right.

described in *Materials and Methods*. Fig. 3 presents the results of such an assay. Both gp160 and gp120 are apparent in OKT4 lanes but not in OKT4A lanes, indicating specific complexing between envelope glycoproteins and CD4. Thus, the mutations introduced into the amino-terminal region of gp41 did not block binding of the mutant envelope glycoproteins to CD4. The variations in amounts of gp160 and gp120 evident in Fig. 3 reflect variations in amounts of envelope glycoproteins expressed by different mutants in this particular assay. Such variations are also apparent in Fig. 2.

DISCUSSION

A number of studies have suggested a role in cell fusion for the hydrophobic region found at the amino termini of the transmembrane envelope glycoproteins of several families of enveloped viruses. These enveloped viruses include the orthomyxoviruses, paramyxoviruses, and retroviruses (for reviews, see refs. 41 and 42). To confirm the involvement in cell fusion of the hydrophobic amino terminus of the HIV-1 gp41 TM glycoprotein, to determine the extent of the region involved in cell fusion, and to examine a possible role in cell fusion of the polar border of the hydrophobic region, we introduced AA substitutions throughout the hydrophobic amino terminus of gp41 and in the polar border of this region. The mutations were then examined for their effects on cell fusion, envelope glycoprotein processing, transport, secretion, and CD4 receptor binding. Use of two envelope expression constructs (a high and a low expressor) in these studies enabled us to distinguish those mutations having effects that could be overcome by increased expression. A summary of the effects of the gp41 mutations appears in Table 2.

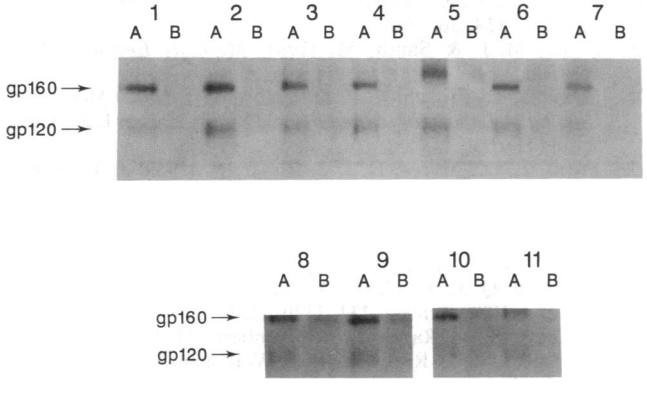

FIG. 3. CD4-binding assay. First immunoprecipitation was performed with OKT4 (lanes A) or OKT4A (lanes B); second immunoprecipitation was performed with AIDS patient sera. Lanes: 1, wild type; 2, mutant 41.1; 3, mutant 41.2; 4, mutant 41.9; 5, mutant 41.26; 6, mutant 41.28; 7, mutant 41.1B/8; 8, wild type; 9, mutant 41.15; 10, mutant 41.29; 11, mutant 41.31. Positions of gp160 and gp120 are indicated.

Polar substitutions at gp41 AAs 2, 9, 15, and 26 completely abrogated the ability of the HIV-1 envelope glycoproteins to induce syncytium formation, whereas polar substitutions at AAs 1 and 28 greatly reduced but did not completely eliminate syncytium formation. A simultaneous double conservative mutation at gp41 AA 1 and 8 also reduced but did not abolish cell fusion. In the polar border of the gp41 hydrophobic amino terminus, a nonconservative mutation at AA 29 completely abrogated syncytium formation, whereas a nonconservative mutation at AA 31 reduced but did not abolish cell fusion.

Any defects in receptor binding would be expected to significantly affect syncytium formation. To determine whether or not receptor-binding defects were contributing to the inability of the mutants to induce syncytium formation, we tested the CD4-binding properties of all of the mutant

Table 2. Effect of gp41 mutation

Mutation*	Change	Syncytium formation	gp160 processing	gp120 secretion†	CD4 binding
Wild type	—	+	+	+	+
gp41.1	Ala → Glu	±	+	+	+
gp41.2	Val → Glu	−	+	+	+
gp41.9	Leu → Arg	−	+	+	+
gp41.15	Ala → Glu	−	+	++	+
gp41.26	Leu → Arg	−	+	++	+
gp41.28	Val → Glu	±	+	+	+
gp41.29	Gln → Leu	−	+	++	+
gp41.31	Arg → Gly	±	+	+	+
gp41.1B/8	Ala → Val Phe → Leu	±	+	+	+

*Number indicates AA position mutated.
†+, Wild-type levels of extracellular gp120; ++, ratio of extracellular to cell-associated gp120 higher than wild-type levels.

envelope glycoproteins. Fig. 3 indicates that none of the mutations blocked the ability of gp160 and gp120 to bind the CD4 receptor molecule.

Because mutations at gp41 AAs 1, 2, 9, and 28 block or dramatically reduce syncytium formation without having any significant effect on gp160 processing, gp120–gp41 association, or CD4 binding, we hypothesize that the entire hydrophobic amino terminus of gp41 is involved in the cell-fusion properties of HIV-1 envelope glycoproteins. The carboxyl-terminal region of this hydrophobic stretch may also be involved in gp120–gp41 association. This latter observation is consistent with previous results (19) indicating that linker insertion mutations in this region (after gp41 AAs 19 and 26) interfere with gp120–gp41 association.

Mutations in the polar border of the hydrophobic amino terminus of gp41 also affected syncytium formation. For the 41.29 mutation, this defect in fusion may, at least partly, result from the increased loss of gp120 from the cell surface and in the decrease in cell-associated gp41. The 41.31 mutation, which decreases syncytium formation, does not affect any aspect of envelope glycoprotein processing or transport examined in these studies. The polar border may therefore play a role in fusion-peptide function, perhaps by acting as a hinge region that facilitates the changes in fusion-peptide conformation necessary for the fusion reaction to occur. A previous study (16) indicated that a mutation in the polar border of the fusion peptide of influenza hemagglutinin decreased syncytium formation.

Further mutational analysis of retroviral fusion peptides will provide more information about the specific requirements for a functional fusion domain and will help elucidate the mechanism by which viral envelope glycoproteins induce syncytium formation. This question is of particular interest and importance in the study of HIV because syncytium formation may contribute to the depletion of $CD4^+$ helper T cells that characterizes AIDS.

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