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Identification of the Principal Neutralizing Determinant of Human Immunodeficiency Virus Type 1 as a Fusion Domain

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The V3 loop, located near the middle of the surface envelope glycoprotein gp120, is the major neutralizing domain of human immunodeficiency virus type 1 (HIV-1). Although the majority of the V3 loop is highly variable between different strains of HIV-1, a Gly-Pro-Gly-Arg motif at the tip of the loop is highly conserved. To determine whether this region plays a role in fusion mediated by the HIV-1 envelope glycoproteins, we introduced seven single-amino-acid changes in the V3 loop. The mutant envelope glycoproteins were expressed from an HIV-1 envelope expression vector and analyzed for their ability to induce cell fusion in the absence of virus replication. Our results indicated that single-amino-acid changes in the V3 loop were capable of completely abolishing or greatly reducing the ability of the HIV-1 envelope glycoproteins to induce cell fusion, thereby identifying the V3 loop as a fusion domain of HIV-1. Mutations in the highly conserved tip of the loop or in a nonconserved region flanking the highly conserved tip had no effect on envelope glycoprotein synthesis, processing, transport, or binding to the CD4 receptor molecule. Mutation of the putative disulfide bridge-forming Cys at residue 336 blocked gp160 cleavage and CD4 binding.

The envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) are synthesized as a polyprotein precursor, gp160, which is cleaved by a host protease to generate the surface envelope glycoprotein gp120 and the transmembrane envelope glycoprotein gp41. The expression of gp120 and gp41 at the plasma membrane can lead to cell-to-cell fusion, or syncytium formation, in appropriate cell types bearing the HIV receptor molecule CD4 (17, 29). The fusion function of the HIV-1 envelope glycoproteins is involved not only in syncytium formation but also in the fusion between the viral envelope and the host cell plasma membrane. This fusion between viral and cellular membranes is an essential step in the infection process. While it is known that cleavage of gp160 is required for the activation of the fusion function (7, 21) and that the hydrophobic amino terminus of gp41 plays a role in the fusion process (6, 8, 15), little is known about other domains of the envelope glycoproteins involved in fusion or the mechanism by which fusion reactions occur.

Several studies have demonstrated that the primary neutralizing domain of HIV-1 lies within a putative loop region located near the middle of gp120 (10, 11, 18, 20, 24, 25). This loop, located in gp120 variable region 3 (V3), is thought to be formed by disulfide bridge formation between Cys residues at gp120 amino acids 301 and 336. Although most of the amino acids of the V3 loop are highly variable between different strains of HIV-1, a Gly-Pro-Gly-Arg sequence at the tip of the loop is highly conserved. The region encompassing this highly conserved tip forms the binding site for antibodies that block HIV-1 infection and inhibit the fusion of HIV-1-infected cells (14, 20, 24, 25, 28).

To test directly whether the V3 loop is involved in syncytium formation mediated by the HIV-1 envelope glycoproteins and to identify residues involved in syncytium formation, we used oligonucleotide-directed mutagenesis to introduce a number of single-amino-acid changes in the V3 loop. The use of an HIV-1 envelope glycoprotein expression system enabled us to analyze, in the absence of viral replication, the effects of the V3 loop mutations on syncytium formation, envelope glycoprotein expression, processing, transport, and CD4 binding.

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MATERIALS AND METHODS

Expression of HIV-1 envelope glycoproteins. To express the envelope glycoproteins of HIV-1, we used the construct pHenv, which we have described previously (7). pHenv was constructed by cloning the *Sal*I-*Bam*HI region of the full-length HIV-1 proviral clone pNL4-3 (1) adjacent to the 5′ HIV-1 long terminal repeat provided by pIIIenv3-1 (29). pHenv thus carries intact *env*, *tat*, and *rev* open reading frames. When transfected into the $CD4^+$ HeLa cell line HeLa T4 (19), pHenv induces the formation of approximately 800 syncytia per μg of transfected DNA (7). pHenv is currently available through the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

DNA mutagenesis and cloning. The *Kpn*I-*Hin*dIII fragment from the HIV-1 proviral clone pNL4-3 (1) was introduced into M13 and subjected to oligonucleotide-directed mutagenesis as described before (16). After mutagenesis, the 300-bp *Bgl*II-*Bsu* 361 fragments containing the V3 loop mutations were introduced into the HIV-1 envelope glycoprotein expression vector pHenv (7). The entire *Bgl*II-*Bsu* 361 fragment was then sequenced by the dideoxynucleotide chain termination method. In all cases, only the desired mutation was present.

Cell culture and fusion assays. The HeLa T4 cell line (19) was cultured as described previously (7, 8) at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% calf serum (HyClone) and G418 (900 μg/ml; GIBCO). For transfections, cells were plated at 8×10^5 cells per 60-mm gridded dish and cultured as above without G418. Calcium phosphate transfections were performed as previously described (9). Two days after transfection with 15 μg of plasmid DNA, the plates were stained, and syncytia (giant

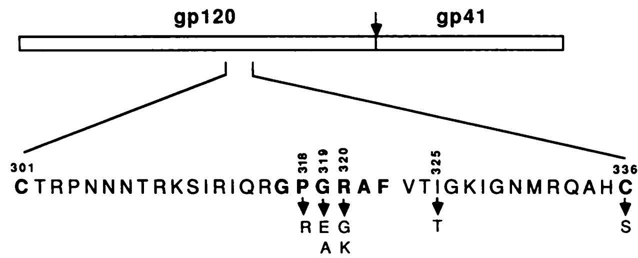

FIG. 1. Mutations introduced into the V3 loop of human immunodeficiency virus gp120. The wild-type sequence is indicated, with the amino acid position numbers shown above the mutated residues. The mutated amino acid residues are shown below the wild-type sequence. The arrow between gp120 and gp41 indicates the site of gp160 cleavage. Single-letter amino acid code: P, Pro; G, Gly; R, Arg; E, Glu; A, Ala; K, Lys; I, Ile; T, Thr; C, Cys; S, Ser.

cells containing more than four nuclei) were scored microscopically.

Cell labeling and radioimmunoprecipitation. Two days after transfection with wild-type or mutant pHenv envelope glycoprotein expression vectors, cells were metabolically labeled with [^{35}S]methionine, and lysates were prepared and immunoprecipitated with serum from a patient with AIDS as described before (7, 9). Sera from AIDS patients were kindly provided by R. Tomar, University of Wisconsin. Immunoprecipitates were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, followed by fluorography.

CD4 binding assay. The CD4 binding assay has been described previously (8). Briefly, 2 days after transfection of HeLa T4 cells with wild-type or mutant pHenv envelope glycoprotein expression vectors, cells were metabolically labeled with [^{35}S]methionine, and lysates were prepared and immunoprecipitated first with anti-CD4 monoclonal antibody OKT4 or OKT4A (Ortho Diagnostic Systems, Inc., Raritan, N.J.), and then with AIDS patient sera containing high titers of anti-HIV-1 envelope antibodies. OKT4 is capable of binding complexes between CD4 and the envelope glycoproteins of HIV-1; thus, gp160 and gp120 are precipitated by OKT4 in the first immunoprecipitation owing to their association with CD4. Since OKT4A competes with gp160 and gp120 for CD4 binding, OKT4A cannot precipitate CD4-envelope glycoprotein complexes (26). The use of these two anti-CD4 monoclonal antibodies thus determines the specificity of envelope glycoprotein binding to CD4.

RESULTS

Mutagenesis of the V3 loop of gp120. To analyze the role of the V3 loop of gp120 in syncytium formation, we used oligonucleotide-directed mutagenesis to introduce a number of single-amino-acid changes in the V3 loop (Fig. 1). In the highly conserved tip of the loop, we changed the Pro at amino acid 318 to Arg (318PR), the Gly at amino acid 319 to both Glu (319GE) and Ala (319GA), and the Arg at amino acid 320 to both Gly (320RG) and Lys (320RK). In a nonconserved portion of the loop, we mutated amino acid 325 from Ile to Thr (325IT). We also mutated the putative disulfide bond-forming Cys at amino acid 336 to Ser (336CS).

Effects of V3 loop mutations on syncytium formation. Following mutagenesis, the mutated *env* genes were introduced into the HIV-1 envelope glycoprotein expression vector pHenv (7) and transfected into the CD4^{+} HeLa cell line HeLa T4 (19). Since the HeLa T4 cell line produces easily quantifiable syncytia upon expression of HIV-1 envelope glycoproteins, it is well suited for the analysis of HIV-1 fusion mutants (6–8). Transfection and expression of the mutant envelope glycoprotein expression vectors in HeLa T4 cells indicated that the 318PR mutation reduced syncytium formation by approximately 90%, the 319GE and 320RG mutations completely abolished syncytium formation, and the 319GA and 320RK mutations reduced syncytium formation by approximately 90 and 98%, respectively. The 325IT mutation reduced syncytium formation by approximately 50%, while the 336CS mutation completely blocked fusion in the HeLa T4 cell line (Table 1).

TABLE 1. Effects of V3 loop mutations on syncytium formation[a]

Mutation	Change	Relative syncytium formation (% of control)
None (wild-type control)		100
318PR	Pro→Arg	12
319GE	Gly→Glu	<1
319GA	Gly→Ala	10
320RG	Arg→Gly	<1
320RK	Arg→Lys	2
325IT	Ile→Thr	44
336CS	Cys→Ser	<1

[a] All data represent averages of at least six assays.

Expression of mutant envelope glycoproteins. As a measure of envelope glycoprotein expression, processing, and transport, cells transfected with wild-type or mutant pHenv vectors were metabolically labeled, lysed, and immunoprecipitated with AIDS patient sera containing high titers of anti-envelope glycoprotein antibodies (Fig. 2). The mutations at positions 318, 319, 320, and 325 had no significant effect on envelope glycoprotein synthesis or processing in the HeLa T4 cell line. The 336CS mutation, however, blocked clevage of gp160 to gp120 and gp41, suggesting that the elimination of disulfide bridge formation significantly altered envelope glycoprotein conformation. Since the HIV-1 envelope glycoproteins are heavily glycosylated dur-

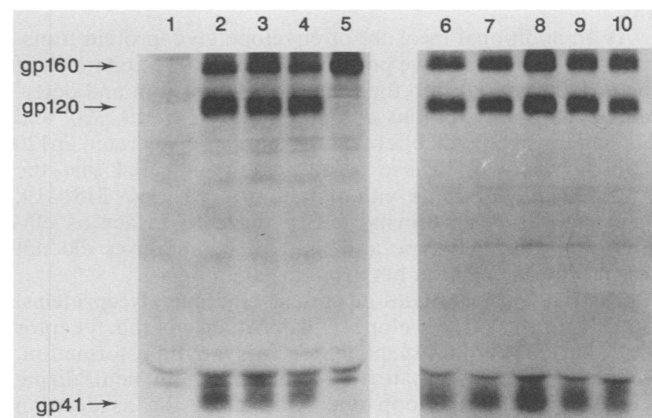

FIG. 2. Immunoprecipitation of mutant envelope glycoproteins. Cells were transfected with no DNA (lane 1), pHenv (lanes 2 and 6), pHenv318PR (lane 3), pHenv320RG (lane 4), pHenv336CS (lane 5), pHenv319GE (lane 7), pHenv319GA (lane 8), pHenv320RK (lane 9), and pHenv325IT (lane 10). The positions of gp160, gp120, and gp41 are indicated.

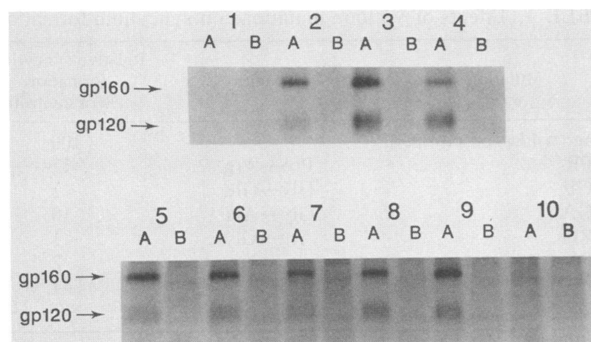

FIG. 3. CD4-binding properties of mutant envelope glycoproteins. The first immunoprecipitation was performed with OKT4 (lanes A) or OKT4A (lanes B). Cells were transfected with no DNA (lanes 1), pHenv (lanes 2 and 5), pHenv318PR (lanes 3), pHenv320RG (lanes 4), pHenv319GE (lanes 6), pHenv319GA (lanes 7), pHenv320RK (lanes 8), pHenv325IT (lanes 9), and pHenv336CS (lanes 10). The positions of gp160 and gp120 are indicated.

ing their transport to the cell surface (23), any significant alteration in transport would result in changes in the apparent molecular weights of the mutant envelope glycoproteins. As is evident in Fig. 2, the mutations at amino acids 318, 319, 320, and 325 did not affect the mobility of the mutant envelope glycoproteins, indicating that these mutations had no significant effect on envelope transport in the HeLa T4 cell line.

gp120 is released in significant amounts from the surface of cells expressing HIV-1 envelope glycoproteins (27). Mutations that interfere with the association between gp120 and gp41 result in an increase in secreted gp120 and a decrease in cell-associated gp120 (8, 15). The mutations at amino acid residues 318, 319, 320, and 325 had no detectable effect on the cell-associated gp160/gp120 ratio (Fig. 2), demonstrating that these mutations did not disrupt the association between gp120 and gp41. To quantify the amounts of gp160 and gp120 present in the immunoprecipitations presented in Fig. 2, the bands from these gels were excised and counted by liquid scintillation. The gp160/gp120 ratios obtained were: pHenv, 1.0; pHenv318PR, 0.9; pHenv320RG, 1.0; pHenv319GE, 1.0; pHenv319GA, 1.2; pHenv320RK, 1.0; and pHenv325IT, 1.0.

As an additional measure of envelope glycoprotein transport and association, we performed immunoprecipitations of the gp120 released into the extracellular medium and determined that the mutations at amino acids 318, 319, 320, and 325 had no significant effect on the amount of secreted gp120 (data not shown). These results again indicated that the envelope glycoproteins with mutations at positions 318, 319, 320, and 325 were transported to the cell surface as efficiently as the wild type and that these mutations did not disrupt the association between gp120 and gp41.

CD4-binding properties of mutant envelope glycoproteins. Binding of HIV-1 envelope glycoproteins to the receptor molecule CD4 is an essential step in syncytium formation. Previous studies indicate that the binding of neutralizing antibody to the V3 loop does not block the association between HIV-1 envelope glycoproteins and CD4, suggesting that the V3 loop is not part of the CD4-binding domain (28). To confirm this result, we performed CD4-binding assays with cells transfected with wild-type or mutant envelope glycoproteins. Representative CD4-binding assays (Fig. 3) demonstrated that the 318PR, 320RG, 319GE, 319GA, 320RK, and 325IT mutations had no effect on CD4 binding, while the 336CS mutation resulted in an envelope glycoprotein that was defective in its ability to bind CD4.

DISCUSSION

The major neutralizing domain of HIV-1 lies within a variable region located near the middle of the surface envelope glycoprotein gp120. This region, known as V3, is believed to form a loop as a result of disulfide bonding between Cys residues at amino acid positions 301 and 336. An eight-amino-acid synthetic peptide homologous to the sequence at the tip of the V3 loop elicited the production in goats of antibodies capable of neutralizing HIV-1 infection in a strain-specific fashion (14). A 15-amino-acid peptide homologous to the tip of the V3 loop bound neutralizing antibody in serum from infected chimpanzees; modifications of this peptide led to the conclusion that the residues critical to neutralizing antibody binding were located adjacent to the highly conserved Gly-Pro-Gly-Arg motif (11). Mutations in the V3 loop enable variants of HIV-1 to escape neutralization by an anti-V3 loop antibody (22). A recent study showed that the presence of anti-V3 loop antibodies in HIV-1-infected women correlated with a significantly lower rate of HIV transmission to fetuses in utero (5).

We have shown that single-amino-acid changes in the V3 loop completely abolish or greatly reduce syncytium formation mediated by the envelope glycoproteins of HIV-1 in the HeLa T4 cell line. Mutation of residue 318 from Pro to Arg resulted in a decrease in syncytium formation of approximately 90%. A conservative change at residue 319 from Gly to Ala also resulted in a 90% decrease in syncytium formation, while a nonconservative change (Gly to Glu) at the same residue led to a complete block in fusion. A recent report also demonstrated that mutation of this highly conserved Gly (to Trp) blocked syncytium formation (13). A conservative mutation at residue 320 (Arg to Lys) decreased fusion by 98%. A nonconservative change at the same residue (Arg to Gly) completely abolished syncytium formation. In the nonconserved region flanking the highly conserved tip of the V3 loop, mutation of amino acid 325 from Ile to Thr led to a decrease in syncytium formation of approximately 50%. None of the mutations introduced into the loop detectably affected envelope synthesis, processing, transport, secretion, or binding to the CD4 receptor molecule. Mutation of the putative disulfide bridge-forming Cys at gp120 amino acid 336 completely abolished syncytium formation, blocked cleavage of gp160 to gp120 and gp41, and impaired envelope glycoprotein binding to CD4. This result is consistent with those of a recent study in which a number of gp120 Cys residues were mutated (32).

Since it is essential to the infection process, membrane fusion plays a central role in the life cycle of HIV-1 and other enveloped viruses. Furthermore, cell fusion has been proposed as a mechanism by which HIV-1 infection results in the depletion of the $CD4^+$ class of T-helper cells in patients with AIDS (for review, see reference 10). This study identifies the V3 loop as a region of gp120 that is involved in the fusion function of HIV-1. This study further demonstrates that the involvement of the V3 loop in syncytium formation is extremely sequence specific. Even very subtle amino acid changes (Gly to Ala or Arg to Lys) result in almost complete abrogation of syncytium formation. The highly conserved tip of the V3 loop is more sensitive to change than the fusion domain at the amino terminus of gp41, as we and others have

shown that conservative changes in this region do not greatly affect syncytium formation (2, 8).

Our observation that a conservative mutation at a nonconserved residue of the V3 loop (amino acid 325) significantly reduced syncytium formation suggests that the nonconserved regions located adjacent to the highly conserved tip of the V3 loop may play a role in fusion, perhaps by interacting with other regions of the envelope glycoproteins. Previous studies demonstrated a role for nonconserved portions of the V3 loop in neutralizing-antibody binding (11) and suggested the possibility of interactions between a nonconserved V3 loop sequence and other portions of gp120 (33).

There are several models that could account for the involvement of the V3 loop in syncytium formation. The highly conserved tip of the V3 loop may bind to a cell surface molecule distinct from CD4. The existence of such a non-CD4 receptor has been posulated by several groups (3, 4, 19, 31). Several recent reports raise the possibility that a proteolytic cleavage event takes place near the tip of the V3 loop and that this cleavage event is required at some stage in the infection process (12, 30). The site of this putative cleavage reaction is thought to be adjacent to the Arg at gp120 amino acid 320, which we mutated to both Gly and Lys. Another model for V3 loop function is that it plays a role in syncytium formation by interacting with other regions of the envelope glycoproteins or by interacting with CD4 in some manner required for the initiation of the fusion reaction. Studies are in progress in our laboratory to distinguish between these and other possible models for V3 loop function.

The results of this study provide insight into the fusion process by defining and characterizing a region of gp120 involved in syncytium formation. Because of its role in membrane fusion, the V3 loop represents a region against which anti-HIV therapeutic agents could be targeted.

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