Effects of Amino Acid Changes in the Extracellular Domain of the Human Immunodeficiency Virus Type 1 gp41 Envelope Glycoprotein

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Changes were introduced into conserved amino acids within the ectodomain of the human immunodeficiency virus type 1 (HIV-1) gp41 transmembrane envelope glycoprotein. The effect of these changes on the structure and function of the HIV-1 envelope glycoproteins was examined. The gp41 glycoprotein contains an amino-terminal fusion peptide (residues 512 to 527) and a disulfide loop near the middle of the extracellular domain (residues 598 to 604). Mutations affecting the hydrophobic sequences between these two regions resulted in two phenotypes. Some changes in amino acids 528 to 562 resulted in a loss of the noncovalent association between gp41 and the gp120 exterior glycoprotein. Amino acid changes in other parts of the gp41 glycoprotein (residues 608 and 628) also resulted in subunit dissociation. Some changes affecting amino acids 568 to 596 resulted in envelope glycoproteins partially or completely defective in mediating membrane fusion. Syncytium formation was more sensitive than virus entry to these changes. Changes in several amino acids from 647 to 675 resulted in higher-than-wild-type syncytium-forming ability. One of these amino acid changes affecting tryptophan 666 resulted in escape from neutralization by an anti-gp41 human monoclonal antibody, 2F5. These results contribute to an understanding of the functional regions of the HIV-1 gp41 ectodomain.

Human immunodeficiency virus type 1 (HIV-1) is the cause of AIDS, which is characterized by the depletion of CD4-positive lymphocytes (6, 26, 33). HIV-1 exhibits a tropism for CD4-bearing cells that is mediated by a specific interaction between the viral envelope glycoproteins and the CD4 glycoprotein (18, 44, 53, 55). The HIV-1 envelope glycoproteins are synthesized as a 160-kDa precursor (gp160) that is cleaved into the exterior envelope glycoprotein (gp120), which binds to the CD4 molecule, and a transmembrane glycoprotein (gp41) (1, 70, 82). The HIV-1 gp120 and gp41 glycoproteins remain associated through noncovalent interactions (38, 46, 56). Following CD4 binding, the envelope glycoproteins mediate a pH-independent process that results in the fusion of the viral and target cell membranes and that is necessary for virus entry (37, 77). HIV-1 envelope glycoproteins expressed on the surface of infected cells initiate receptor-binding and membrane fusion events with adjacent CD4-positive cells, resulting in the formation of multinucleated syncytia (52, 75).

Structural and mutagenic studies have provided some insight into the molecular events underlying HIV-1 entry and syncytium formation. The CD4-binding region of gp120 consists of a conformation-dependent structure with elements derived from discontinuous sequences in the third and fourth conserved regions of the glycoprotein (16, 17, 46, 50, 62). The gp120 glycoprotein binds to the CD4 region bearing similarity to the second complementarity-determining (CDR2) region of immunoglobulins (3, 4, 7, 11, 12, 14, 15, 42, 49, 57, 66). The CDR2-like region of CD4, located in the amino-terminal domain of the glycoprotein, consists of a hydrophobic ridge protruding from a positively charged surface (73, 83). Mutagenic studies have indicated that the gp120-binding

site is discontinuous but spatially limited to the CD4 region near phenylalanine 43, lysine 46, and arginine 59 (14).

The events that, in addition to CD4 binding, are necessary for membrane fusion are poorly understood. Amino acid changes in specific regions of both the gp120 and gp41 glycoproteins result in molecules that are structurally wild type and retain receptor-binding ability but are fusion defective. These regions include the third variable (V3) region, which is a target for neutralizing antibodies, and other regions of the gp120 glycoprotein (10, 29, 35, 37, 41, 43, 54, 63, 69, 72, 79, 81). Alteration of two regions of the gp41 glycoprotein, the hydrophobic amino terminus and the membrane-spanning region, has resulted in fusion-defective phenotypes (10, 28, 37, 39, 46). The analogous position of hydrophobic, fusogenic domains on the amino termini of transmembrane glycoproteins of several enveloped viruses suggests functional similarities in the membrane fusion process (for reviews, see references 84 and 85). It has been proposed that for viruses such as HIV-1 that fuse membranes at neutral pH, binding to the viral receptor predisposes to conformational changes in the envelope glycoproteins analogous to those induced by acidic pH, which triggers the entry of orthomyxoviruses (2, 8, 13, 46, 60, 74, 76, 84, 85). In the latter case, these changes result in exposure of the hydrophobic fusion peptide at the amino terminus of the HA₂ transmembrane glycoprotein, perhaps favoring HA₂ interaction with the membrane(s) involved in the fusion process (76, 84, 85). Recent studies have provided genetic evidence that the amino terminus of the HIV-1 gp41 glycoprotein may interact with the target cell during the membrane fusion event (10). A number of studies have suggested that interaction with the CD4 glycoprotein can cause conformational alterations in the HIV-1 envelope glycoproteins, including dissociation of the gp120 glycoprotein from the gp41 glycoproteins (2, 8, 13, 36, 59, 60, 74), but

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the importance of these events to the membrane fusion process remains unclear (9, 58, 79). The gp41 glycoprotein of HIV-1, in the absence of the gp120 glycoprotein, has been reported to retain membrane-fusing capacity (65).

The membrane-spanning region of the HIV-1 transmembrane envelope glycoprotein and those of some other lentiviruses exhibit an unusual structure, in which three hydrophobic stretches are interrupted by two positively charged residues (39). Changes affecting these two charged residues significantly affect the efficiency of the membrane fusion process (39).

Here the effects of changes in conserved amino acids of the HIV-1 gp41 ectodomain on the structure and function of the envelope glycoproteins are examined.

MATERIALS AND METHODS

Construction of mutants. Mutations were introduced into the HIV-1 (HXBc2 strain) *env* sequence by the site-directed mutagenesis procedure of Kunkel et al. (48). Mutated *env* fragments were selected from two independent clones to minimize the possibility that spontaneous mutations distant from the desired site contribute to the observed phenotype. Mutated *env* fragments were expressed in the pSVIIIenv plasmid as described previously (37, 62).

Envelope glycoprotein expression and CD4-binding assay. COS-1 cells were transfected by the DEAE-dextran method with pSVIIIenv DNA expressing wild-type or mutant envelope glycoproteins as described previously (37, 62). In some experiments, a *tat*-expressing plasmid was cotransfected to increase the level of envelope glycoprotein expression. To measure expression, the cells were radiolabeled with [³⁵S]cysteine overnight and precipitated with an excess of a mixture of sera derived from AIDS patients (39, 62). Expression of the mutant glycoproteins, processing of the glycoprotein precursor, association of gp41 and gp120 glycoproteins, and ability of the supernatant gp120 to bind CD4-positive SupT1 lymphocytes were determined as described previously (62). The pSVIIIenvΔKS plasmid, which contains a frame shift and deletion within the *env* gene, was used as a negative control.

Cell surface expression of envelope glycoproteins. The expression of the mutant glycoproteins on the surface of transfected COS-1 cells was measured by using two assays.

(i) **Radiolabeled anti-gp120 antibody assay.** COS-1 cells were transfected with 10 μg of pSVIIIenv DNA expressing wild-type or mutant envelope glycoproteins. At 60 h after transfection, the COS-1 cells were washed with phosphate-buffered saline (PBS) containing 2% fetal bovine serum and incubated with a ³⁵S-labeled monoclonal antibody against gp120, F105. F105 antibody-producing hybridomas were obtained from Marshall Posner (68), and 10⁸ cells were labeled overnight in 80 ml of cysteine- and methionine-free RPMI 1640 medium containing 5 mCi each of [³⁵S]cysteine and [³⁵S]methionine. A 1-ml portion of the F105 antibody-containing medium was added to each 100-mm dish of transfected COS-1 cells. The envelope-expressing COS-1 cells were incubated with the antibody for 1 h at 37°C, washed three times with PBS containing 2% fetal bovine serum, and lysed. The lysates were incubated with protein A-Sepharose beads, which were washed by the procedure used for immunoprecipitation (62). The labeled antibody was visualized on a reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel.

(ii) **Iodination of cell surface envelope glycoproteins by lactoperoxidase.** For some of the mutants, the envelope

glycoproteins on the cell surface were examined by iodination followed by immunoprecipitation with a mixture of sera from AIDS patients. A 100-mm dish of transfected COS-1 cells was washed four times with ice-cold PBS. Then 900 μl of PBS was added to the dish, followed by 100 μl of lactoperoxidase (100 U/ml in PBS; Calbiochem), 0.5 mCi of ¹²⁵I (Amersham), and 25 μl of a 1:1,000 (vol/vol) solution of 30% hydrogen peroxide in distilled water. An additional 25 μl of the hydrogen peroxide solution was added after 5, 10, and 15 min. After a total incubation time of 20 min at room temperature, the cells were lysed and used for immunoprecipitation with a mixture of sera derived from AIDS patients.

Functional assays for the HIV-1 envelope glycoproteins. The ability of the mutant glycoproteins to mediate the formation of syncytia was assessed by cocultivating transfected COS-1 cells with SupT1 CD4-positive lymphocytes as previously described (80). Complementation of a single round of replication of the *env*-deficient chloramphenicol acetyltransferase (CAT)-expressing provirus by the mutant envelope glycoproteins was performed as described previously (37, 80).

Soluble CD4-induced shedding of gp120. COS-1 cells transfected with the envelope glycoprotein-expressing plasmids were labeled with [³⁵S]cysteine (200 μCi in 5 ml of cysteine-free medium) overnight at 48 h following transfection. The labeled cells were then washed and incubated with either 0, 10, or 30 μg (0, 200, or 600 nM) of soluble CD4 (American BioTechnologies, Inc.) per ml in PBS for 1 h at 37°C. The cell supernatants were then precipitated with a mixture of sera from AIDS patients.

Soluble CD4 binding to cell surface envelope glycoprotein complexes. Transfected COS-1 cells were incubated with 2.4 × 10⁻⁹, 1.2 × 10⁻⁸, or 6 × 10⁻⁸ M soluble CD4 that had been iodinated by the Bolton-Hunter method to a specific activity of 45 Ci/mmol. These concentrations were calculated from the estimated fraction of native soluble CD4 in the iodinated fraction and were chosen to be near the dissociation constant for the soluble CD4-cell surface HIV-1 envelope glycoprotein interaction. The use of these soluble CD4 concentrations allows maximum sensitivity with respect to changes in affinity. Incubations were carried out at 4°C for 3 h, after which the cells were washed three times in PBS, lysed, and immunoprecipitated with OKT4 antibody. Bound soluble CD4 was visualized on an SDS-polyacrylamide gel, and the autoradiograms were quantitated by densitometry.

Virus neutralization assay. For the virus neutralization assay, recombinant virus encoding CAT and containing either wild-type or mutant envelope glycoproteins was produced in COS-1 cells as described previously (37). Virus was incubated with 6 μg of 2F5 antibody per ml or with no antibody at 37°C for 1 h, and then Jurkat lymphocytes were added to the virus preparation. CAT activity was measured in the target cells, and the degree of neutralization was assessed (37, 79).

RESULTS

HIV-1 gp41 glycoprotein mutants. Amino acid changes were introduced into the gp41 glycoprotein of the HIV-1 (HXBc2 strain) isolate by site-directed mutagenesis (48). Most of the amino acids selected for alteration are well conserved among the different primate immunodeficiency viruses and were changed in a nonconservative manner. The mutated *env* genes were expressed in COS-1 cells, and the precursor-processing ability, gp120-gp41 association ability, CD4-binding ability, syncytium-forming activity, and ability

TABLE 1. Phenotypes of HIV-1 gp41 mutants

Envelope glycoprotein	Processing index ^a	Association index ^a	Cell surface expression ^b :		Soluble CD4-induced dissociation ^c	Syncytium formation	Replication complementation		
			F105 binding	¹²⁵ I labeling			SupT1	C8166	Jurkat
Wild type	1.00	1.00	+++	+++	+++	100	100	100	100
528 S/T	0.61	0.21	+	ND ^d	++	0-4	35	17	14
530 M/S	0.21	0	+++	+++ (gp160)	+	0	0.9	0.8	0
548 I/A	0.66	1.62	+++	+++	+++	7-23	74	113	56
552 Q/L	0.10	0	ND	ND	ND	0	ND	ND	ND
555 L/G	0.18	0	ND	ND	ND	0	ND	ND	ND
562 Q/L	0.28	0.12	ND	ND	ND	0	ND	ND	ND
566 L/G	0.29	0.55	++	ND	-	0	3	1	10
568 L/A	0.62	0.98	+++	+++	+++	0	42	30	15
571 W/R	0.49	0.87	+++	+++	-	0	2	0.7	0
584 E/A	0.12	0	+++	ND	+	0	ND	ND	0
589 D/L	1.9	0.44	+++	+++	++	0	5	1	8
596 W/M	1.06	0.60	+++	+++	+++	0	105	100	108
608 V/S	0.20	0	ND	ND	ND	0	ND	ND	ND
626 T/M	0	NA ^e	-	—	-	0	0.5	0	4
628 W/M	0.10	0	ND	ND	ND	0	ND	ND	ND
639 T/A	0	NA	-	-	-	0	ND	ND	ND
647 E/L	1.30	0.43	+++	ND	ND	84-110	ND	ND	95
652 Q/L	1.03	0.67	+++	ND	ND	225-230	ND	ND	99
656 N/L	0.62	0.78	+++	+++	+++	0	12	9	9
663 L/F	1.53	0.80	+++	ND	ND	152-190	ND	ND	94
666 W/P	0.96	1.02	+++	ND	ND	154-190	ND	ND	48
669 L/P	1.02	0.56	++++	ND	++++	220-260	93	81	49
671 N/P	0.82	1.09	+++	ND	ND	185-193	ND	ND	70
672 W/S	1.03	0.74	+++	ND	++++	208-260	121	122	94
673 F/P	1.13	0.42	+++	ND	ND	108-150	ND	ND	70
675 I/S	1.24	0.54	+++	ND	ND	102-130	ND	ND	73
677 N/R	0.93	0.57	+++	ND	ND	25-41	ND	ND	81
678 W/A	1.13	0.60	+++	ND	ND	93-105	ND	ND	ND
681 Y/P	0.99	0.77	ND	ND	ND	67-74	ND	ND	ND

^a To calculate the processing index, the amounts of gp160 and gp120 glycoproteins were determined by densitometric scanning of autoradiograms of SDS-polyacrylamide gels as follows: processing index = ([total gp120]_{mutant} × [gp160]_{wild-type}) / ([gp160]_{mutant} × [total gp120]_{wild-type}). The association index indicates the degree of association between the mutant gp120 and gp41 glycoproteins, relative to that of wild-type glycoproteins. The association index was calculated as follows: association index = ([mutant gp120]_{cell} × [wild-type gp120]_{supernatant}) / ([mutant gp120]_{supernatant} × [wild-type gp120]_{cell}).

^b Cell surface expression was evaluated by using F105 binding or iodination. Symbols: +++++, levels 1.5- to 2-fold higher than wild type; +++, wild-type levels; ++, 50 to 90% of wild-type levels; +, 10 to 49% of wild-type levels; -, <10% of wild-type levels.

^c Symbols: +++++, greater than wild-type levels of gp120 shed in response to all concentrations of soluble CD4; +++, wild-type levels of shedding; ++, shedding occurs at slightly lower than wild-type levels; +, shedding occurs but at significantly reduced levels compared with that of wild-type; -, no observed shedding of gp120 at 30 µg of soluble CD4 per ml.

^d ND, not determined.

^e NA, not applicable.

^f Only gp160 was detected at low levels on the cell surface.

to complement virus entry were assessed (Table 1; Fig. 1). Five phenotypes were observed and are discussed accordingly.

Envelope glycoproteins exhibiting defects in membrane fusion. Five mutant envelope glycoproteins (568 L/A, 571 W/R, 589 D/L, 596 W/M, and 656 N/L) exhibited substantial levels of gp160 precursor processing and gp120-gp41 association, yet failed to form syncytia with CD4-positive human lymphocyte lines. A sixth mutant, 548 I/A, was less efficient than the wild-type glycoproteins in the formation of syncytia. The relative pattern of syncytium-forming ability of the mutants was similar with C8166, SupT1, and Jurkat target cells, although, as previously reported (10), syncytium formation was most efficient for both the wild-type and mutant glycoproteins with C8166 target cells (data not shown). The level of expression of these mutant glycoproteins on the transfected COS-1 cell surface was comparable to that of the wild-type glycoprotein, as judged both by binding of a radiolabeled anti-gp120 antibody, F105 (68), to the transfected-cell surface and by iodination of cell surface proteins. These results indicate that any changes in total gp120 glyco-

protein in the transfected cell lysate as a result of slight alterations in precursor processing or subunit association did not significantly affect the level of mutant glycoproteins on the cell surface. The binding of the soluble gp120 glycoprotein to the CD4 receptor on SupT1 cells was unaffected by any of the mutations (data not shown). In addition, with the exception of the 571 W/R mutant, all of these fusion-defective mutants exhibited levels of gp120 dissociation in response to soluble CD4 equivalent to that of the wild-type envelope glycoproteins (Table 1; Fig. 2). These results indicate that, for most of these mutants, the multimeric envelope glycoprotein complex on the cell surface can bind CD4 efficiently (79). Thus, the defectiveness for syncytium formation observed for these mutants cannot be explained by obvious changes in cell surface expression or in CD4-binding ability. By contrast, the 571 W/R mutant did not exhibit soluble CD4-induced shedding of the gp120 glycoprotein, and the binding of radiolabeled soluble CD4 to COS-1 cells expressing the 571 W/R mutant was reduced compared with that observed for cells expressing the wild-type glycoproteins (Fig. 3).

FIG. 1. Expression of HIV-1 wild-type or mutant envelope glycoproteins in COS-1 cells. Radioimmunoprecipitation of envelope glycoproteins from COS-1 cell lysates and supernatants with a mixture of sera from AIDS patients. The names of the mutant glycoproteins appear above the lanes.

The ability of the five mutant envelope glycoproteins identified as fusion deficient to complement virus entry into different target cells was examined. The amino acid changes generally affected the virus entry function less than they affected the syncytium-forming ability. For example, the ability of 548 I/A and 596 W/M mutants to mediate virus entry into three different lymphocyte lines (SupT1, C8166, and Jurkat cells) was almost equivalent to that of the wild-type glycoproteins. However, these mutants were partially (for the 548 I/A mutant) or almost completely (for the 596 W/M mutant) defective in inducing syncytia in these target cells. Similarly, the 568 L/A mutant was completely defective for syncytium formation, even though 15 to 42% of wild-type replicative ability was observed in the different target cell lines. Mutants 566 L/G, 571 W/R, and 589 D/L exhibited a significant reduction in ability to complement virus entry in the lymphocyte lines tested.

Envelope glycoprotein mutants exhibiting decreased gp120-gp41 association. Eight gp41 mutants (528 S/T, 530 M/S, 552 Q/L, 555 L/G, 562 Q/L, 584 E/A, 608 V/S, and 628 W/M) exhibited a phenotype consistent with inefficient association

FIG. 2. Dissociation of gp120-gp41 complexes by soluble CD4. The amount of the gp120 glycoprotein immunoprecipitated from the supernatants of COS-1 cells expressing no HIV-1 envelope glycoproteins (Δ KS), the wild-type HIV-1 envelope glycoproteins, or mutant envelope glycoproteins is shown. Transfected COS-1 cells were incubated with 0, 10, or 30 μ g of soluble CD4 per ml prior to immunoprecipitation.

FIG. 3. Binding of iodinated soluble CD4 to envelope glycoprotein complexes. The amount of 125 I-soluble CD4 bound to the surface of COS-1 cells expressing the wild-type (WT) envelope glycoproteins or the 566 L/G, 571 W/R, or 596 W/M mutants as a function of soluble CD4 (sCD4) concentration is shown. The amount of bound soluble CD4 is expressed in arbitrary units determined by densitometry. Binding to control COS-1 cells transfected with the Δ KS plasmid was undetectable in this range of soluble CD4 concentration.

of the gp120 and gp41 glycoprotein subunits (Fig. 1; Table 1). For these mutants, the cell-associated levels of gp120 glycoproteins were significantly reduced from those observed for the wild-type glycoproteins, but the levels of gp120 glycoprotein spontaneously shed into the cell supernatants were comparable to those of the wild-type glycoproteins. This is reflected in the low association indices calculated for these mutants. As has been observed previously for HIV-1 envelope glycoprotein mutants defective for subunit association (38), the steady-state levels of gp41 in the transfected COS-1 cells were lower than those of the wild-type glycoproteins. Unexpectedly, the cell surface expression, examined by binding the F105 antibody, of some of the association-defective mutants (530 M/S and 584 E/A) was similar to that of the wild-type glycoproteins (Table 1). However, iodination revealed that, in contrast to the wild-type glycoproteins, of which the gp120 glycoprotein was the major form expressed on the cell surface, the gp160 precursor of the 530 M/S mutant was the major cell surface component. Most of the association-defective mutants also exhibited moderate or significant decreases in the level of gp160 precursor processing.

The function of the association-defective mutants was, as expected, decreased. All of the mutants were unable to induce the formation of syncytia. Only one of the mutants, 528 S/T, exhibited the ability to complement virus entry into SupT1 and C8166 lymphocytes, at a level of efficiency lower than that of the wild-type glycoproteins.

Processing-defective gp41 mutants. Two of the amino acid changes (626 T/M and 639 T/A) altered known glycosylation addition sequences. These mutant glycoproteins were completely defective for processing of the gp160 envelope glycoprotein precursor. The gp160 precursor protein of these mutants migrated faster on SDS-polyacrylamide gels than the wild-type gp160 glycoprotein did, consistent with a change in the degree of glycosylation. The 626 T/M and 639 T/A mutant glycoproteins were not efficiently expressed on the transfected COS-1 cell surface, as judged by labeled

FIG. 4. Neutralization of HIV-1 gp41 mutants by the 2F5 antibody. The percent CAT activity observed in Jurkat target cells after incubating the recombinant CAT virus with 2F5 antibody (6 μ g/ml) compared with the activity after incubating the virus in the absence of antibody is shown. A value of 100 represents no neutralization, and a value of 0 represents complete neutralization. The actual values are indicated above the bars.

F105 antibody binding or iodination (Table 1). These mutants did not exhibit detectable syncytium-forming ability.

The 566 L/G mutant exhibited some decreases in precursor processing, and its cell surface expression was slightly decreased compared with that of the wild-type glycoproteins. This mutant did not shed the gp120 glycoprotein in response to soluble CD4 or induce the formation of syncytia (Table 1; Fig. 2). Furthermore, the binding of radiolabeled soluble CD4 to the surface of COS-1 cells expressing the 566 L/G mutant was significantly decreased compared with the binding to the wild-type glycoproteins. Apparently, multiple factors contribute to the poor function of this mutant glycoprotein.

Functional envelope glycoproteins with gp41 amino acid changes. A number of mutant glycoproteins with changes in the carboxyl portion of the gp41 ectodomain (647 E/L, 652 Q/L, 663 L/F, 666 W/P, 669 L/P, 671 N/P, 672 W/S, 673 F/P, 675 I/S, 677 N/R, 678 W/A, and 681 Y/P) exhibited efficient gp160 precursor processing and gp120-gp41 subunit association. These mutant glycoproteins were all capable of inducing the formation of syncytia. In some cases, the syncytium-forming ability was greater than that of the wild-type glycoproteins. For most of the mutants, this increased syncytium-forming activity was not simply a consequence of increased cell surface expression, although an increase in cell surface expression of the 669 L/P mutant was observed (Table 1). Entry into Jurkat, SupT1, and C8166 cells could be efficiently mediated by these mutant glycoproteins.

Escape from neutralization by an anti-gp41 antibody. The 2F5 monoclonal antibody has been reported to neutralize a variety of HIV-1 isolates and recognizes sequences in the gp41 ectodomain near the membrane-spanning region (61). Since most of the HIV-1 mutants with changes in this region exhibited replicative ability comparable to or better than that of the wild-type glycoprotein, the neutralization of these mutants by the 2F5 antibody was examined. Most of the mutants were neutralized by the 2F5 antibody comparably to the wild-type glycoprotein (Fig. 4). The 666 W/P mutant, however, was significantly less sensitive to neutralization by

FIG. 5. Summary of gp41 mutant phenotypes. The ectodomain and membrane-spanning (TM) region of the HIV-1 gp41 glycoprotein is depicted, with the intracytoplasmic (IC) region truncated. The position of the amino-terminal fusion peptide (F) is indicated, as are the disulfide bond (-S-S-), the N-linked glycosylation sites (Y), the epitope recognized by the 2F5 monoclonal antibody, and the positively charged residues proximal to the membrane-spanning region (+). The positions of amino acids in which changes decreased gp120-gp41 subunit association, membrane-fusing ability, or gp160 precursor processing are shown. The positions of residues in which changes resulted in envelope glycoproteins with wild-type or better-than-wild-type fusion activity are also shown.

the 2F5 antibody than was the wild-type glycoprotein or the other mutants tested.

DISCUSSION

The gp41 glycoprotein exterior domain is important for noncovalent association with the gp120 exterior envelope glycoprotein and for membrane fusion. This study provides insight into the structural features of the gp41 ectodomain that contribute to these functions. A summary of the gp41 mutant phenotypes from this study is shown in Fig. 5.

The initial folding, glycosylation, and oligomerization of the HIV-1 gp160 envelope glycoprotein precursor occur in the endoplasmic reticulum (23, 27, 64, 78, 86). It is likely, from studies of misfolded mutant glycoproteins or glycosylation inhibitors, that these processes are necessary for the subsequent movement of the glycoproteins to the Golgi complex, where gp160 cleavage occurs (19, 20). Proper folding and oligomerization have been shown to be important for the intracellular movement of other viral envelope glycoproteins (24, 25, 34, 40, 47, 71). Oligomerization of the HIV-1 envelope glycoproteins depends on sequences in the gp41 ectodomain, but the precise location of these sequences has not been determined (23, 67). Since the HIV-1, HIV-2, and simian immunodeficiency virus SIV_{mac} envelope glycoproteins can form heteromultimers (21), it is likely that these sequences reside in gp41 regions conserved among these viruses. Between the fusion peptide at the amino terminus (residues 512 to 527) and the intramolecular disulfide bond (residues 598 to 604) lies a hydrophobic, conserved sequence that has been proposed to form an amphipathic α -helix (32). Since the remainder of the gp41 ectodomain is less well conserved among the primate immunodeficiency viruses, it is likely that the determinants for oligomerization reside within the region between the fusion peptide and the disulfide bond. Some of the amino acid changes in this region resulted in a decreased efficiency of gp160 precursor cleavage, which may reflect conformational changes and/or decreased efficiency of oligomer formation. Since the amino acids in which changes affected gp160 processing were not localized to a discrete region of the gp41 ectodomain, multiple interactions probably contribute to proper folding

and oligomer formation. A recent study has shown that the leucine zipper-like structure in this region can be altered without affecting oligomerization (22).

A gp41 region (residues 528 to 562) immediately carboxyl to the fusion peptide is apparently important for association with the gp120 envelope glycoprotein. Previous studies have also observed effects on gp120-gp41 association resulting from changes in this region (46). The proximity of a probable gp120 contact region and the fusion peptide provides an explanation for how changes in gp120 conformation might influence the conformation of gp41 amino-terminal sequences important for the membrane fusion reaction. Contacts between gp41 and gp120 are probably not limited to the gp41 region spanning residues 528 to 562, since changes outside this region can affect subunit association. These results are consistent with mutagenic studies of the HIV-1 gp120 glycoprotein, which indicate that at least two hydrophobic stretches in conserved regions C1 and C5 of gp120 contribute to the gp41 interaction, with additional contributions to gp120-gp41 affinity involving other hydrophobic gp120 regions in C3 and C4 (38).

Some changes in gp41 residues 566 to 596 resulted in envelope glycoproteins that were expressed on the cell surface, that bound CD4, but that were defective for syncytium formation. Thus, some aspect of the membrane fusion process appears to be deficient for these mutants. Interactions with other components of the envelope glycoproteins or with the target cell may be affected by these amino acid alterations. With respect to the latter possibility, however, we did not observe patterns of target cell-specific entry or syncytium formation that suggested direct interaction with the target cell, as has been observed for amino acid changes in the gp41 amino terminus (10). Changes in the leucine-rich amphipathic α -helix within this region have been recently reported to result in fusion-defective envelope glycoproteins (22).

In general, virus entry was disrupted less by changes in gp41 residues 566 to 596 than was syncytium formation. This has been previously observed for some fusion-deficient HIV-1 envelope glycoprotein mutants and has been attributed to differences in the number of successful envelope glycoprotein-CD4 interactions required for cell-cell fusion compared with virus-cell fusion (37, 45). This explanation appears sufficient to explain the phenotypes observed for some of the mutants (e.g., 548 I/A, 568 L/A, 589 D/L, and 656 N/L), for which the virus entry process was less efficient than that of the wild-type glycoproteins. For the 596 W/M mutant, however, no defect in the ability to complement virus entry was observed despite a severe defect in syncytium-forming ability. These results suggest that qualitative differences in the gp41 structural requirements for virus entry and syncytium formation exist. The 571 W/R mutant was expressed efficiently on the cell surface but, unlike most of the other fusion-defective envelope glycoproteins, did not exhibit gp120 shedding in response to incubation with soluble CD4. This phenotype has been observed for gp120 mutants that are deficient in CD4 binding or are able to bind soluble CD4 but are deficient in the shedding process *per se* (79). The 571 W/R mutant exhibited decreases in binding of soluble CD4 to the multimeric envelope glycoprotein complex, even though monomeric gp120 binding to CD4-positive cells was not affected by this amino acid change. Apparently, changes in this gp41 amino acid can influence the ability of the envelope glycoprotein complex to bind CD4. This might occur either by a direct effect of the amino acid change on the structure of the CD4-binding region or by a

secondary effect of the amino acid change on the orientation of the gp120 molecules in the multimeric complex, resulting in a steric inhibition of CD4 binding.

Changes in two of the potential sites for N-linked glycosylation dramatically affected the processing and cell surface transport of the gp160 glycoprotein. This processing defect, which probably reflects conformational disruption of the precursor glycoprotein, is probably a result of the particular amino acid substitutions introduced rather than a result of loss of glycosylation at these sites. This conclusion is based on reports that other amino acid changes that affect glycosylation addition at these positions do not abrogate gp160 precursor processing (19, 51).

The HIV-1 gp41 sequences immediately amino terminal to the membrane-spanning region exhibit significant hydrophilic character. Although one mutant (656 N/L) with a change in this region was membrane fusion defective, most of the other mutants with amino acid alterations in this region exhibited syncytium-forming ability comparable to or better than that of the wild-type glycoproteins. It has been previously reported that deletions of the cytoplasmic tail of the HIV-1 gp41 glycoprotein result in increased syncytium-forming ability (5, 30, 46). It is interesting that changes in the gp41 glycoprotein regions adjacent to the exterior side of the membrane can also result in increased fusogenic capacity. The basis for either of these increases remains to be determined, but increases in cell surface expression of the mutants do not adequately account for the increase in syncytium formation.

An amino acid change (666 W/P) in the gp41 region immediately adjacent to the membrane-spanning region resulted in escape from neutralization by the 2F5 monoclonal antibody. Although this region is relatively invariant in natural HIV-1 isolates, these results indicate that changes in this region allowing neutralization escape are well tolerated, with only minor effects on virus replication. The functional integrity of viruses with changes in this region indicates that the neutralizing ability of the 2F5 antibody probably does not result from inhibition of a fusion-related function of its target sequence, in contrast to neutralizing antibodies directed against the gp120 V3 loop (29, 35, 37, 41, 63, 81). The epitope for the 2F5 antibody has been mapped in other studies (61) to residues 662 to 667. The virus-neutralizing activity of the 2F5 antibody suggests that this region is not completely buried within the membrane. The amino-terminal extent of the membrane-spanning region is likely to map between residues 668 and 684, the latter being associated with stop transfer signals in a previous study (31). A potential site of N-linked glycosylation in the HXBc2 envelope glycoprotein at position 674 is apparently not utilized (51), suggesting that its accessibility may be limited by membrane association. Further studies should refine our understanding of the structure and function of the HIV-1 transmembrane envelope glycoprotein.

ACKNOWLEDGMENTS

We thank Mark Kowalski for help with some of the experiments. We thank Virginia Nixon for manuscript preparation and Amy Emmert for artwork.

This work was supported by a grant from the National Institutes of Health (AI24755) to Joseph Sodroski and by a Center for AIDS Research grant to Dana-Farber Cancer Institute. Louise Bergeron was supported by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche.

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