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Functional and Immunologic Characterization of Human Immunodeficiency Virus Type 1 Envelope Glycoproteins Containing Deletions of the Major Variable Regions

RICHARD WYATT,[1] NANCY SULLIVAN,[1] MARKUS THALI,[1] HEINRICH REPKE,[1] DAVID HO,[2] JAMES ROBINSON,[3] MARSHALL POSNER,[4] AND JOSEPH SODROSKI[1]*

Division of Human Retrovirology, Dana-Farber Cancer Institute, Department of Pathology,[1] and Department of Medicine, New England Deaconess Hospital,[4] Harvard Medical School, Boston, Massachusetts 02115; The Aaron Diamond AIDS Research Center for the City of New York, New York University School of Medicine, New York, New York 10016[2]; and Department of Pediatrics, University of Connecticut Health Services, Farmington, Connecticut 06030[3]

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Deletions of the major variable regions (V1/V2, V3, and V4) of the human immunodeficiency virus type 1 (HIV-1) gp120 exterior envelope glycoprotein were created to study the role of these regions in function and antigenicity. Deletion of the V4 region disrupted processing of the envelope glycoprotein precursor. In contrast, the deletion of the V1/V2 and/or V3 regions yielded processed exterior envelope glycoproteins that retained the ability to interact with the gp41 transmembrane glycoprotein and the CD4 receptor. Shedding of the gp120 exterior glycoprotein by soluble CD4 was observed for the mutant with the V3 deletion but did not occur for the V1/V2-deleted mutant. None of the deletion mutants formed syncytia or supported virus entry. Importantly, the affinity of neutralizing antibodies directed against the CD4-binding region for the multimeric envelope glycoprotein complex was increased dramatically by the removal of both the V1/V2 and V3 structures. These results indicate that, in addition to playing essential roles in the induction of membrane fusion, the major variable regions mask conserved neutralization epitopes of the HIV-1 gp120 glycoprotein from antibodies. These results explain the temporal pattern associated with generation of HIV-1-neutralizing antibodies following infection and suggest stratagems for eliciting improved immune responses to conserved gp120 epitopes.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (3, 24). HIV-1 establishes a persistent infection in human hosts, eventually resulting in defective cellular immunity secondary to CD4 lymphocyte depletion (19, 43).

The HIV-1 exterior envelope glycoprotein, gp120, and the transmembrane envelope glycoprotein, gp41, are derived by cleavage of the gp160 envelope glycoprotein precursor (2, 67). HIV-1 is tropic for CD4-positive cells by virtue of a high-affinity interaction between gp120 and the CD4 glycoprotein, which acts as the virus receptor (14, 41, 47, 49). Following gp120-CD4 binding, fusion of viral and host cell membranes, which involves both gp120 and gp41, allows virus entry (31, 74). Similar processes mediate the formation of syncytia between cells expressing the HIV-1 envelope glycoproteins and CD4-positive cells (31, 46, 72).

The HIV-1 envelope glycoproteins possess regions that are highly variable among strains and that are interspersed between conserved regions. Five variable regions (V1/V2, V3, V4, and V5) and five conserved regions (C1 to C5) have been defined on the gp120 exterior envelope glycoprotein (55). The V1/V2, V3, and V4 regions form disulfide-linked loops (45). The gp41 ectodomain and transmembrane region are well conserved among HIV-1 isolates (55).

Mutagenic studies of the HIV-1 envelope glycoproteins have identified some of the regions important for virus entry or syncytium formation. Amino acid changes in gp120 conserved regions C1 and C5, as well as in the gp41 ectodomain, disrupt the noncovalent association of the two envelope glycoprotein subunits, resulting in loss of ability to mediate virus entry or syncytium formation (10, 32, 42). CD4 binding is dependent on gp120 amino acids located in three discontinuous sequences within the C3 and C4 conserved regions (12, 44, 59). These residues, in conjunction with amino acids located in the other conserved gp120 regions, constitute discontinuous epitopes recognized by neutralizing antibodies capable of blocking gp120-CD4 binding (50, 76, 78). Amino acid changes in other specific regions of both the gp120 and gp41 proteins result in envelope glycoproteins that exhibit wild-type precursor processing, subunit association, and receptor binding but that are fusion defective. These regions include the third variable (V3) loop of gp120, which is also a target for neutralizing antibodies (25, 38, 39, 48, 58, 65, 68, 70), the fourth conserved (C4) gp120 region, the gp41 amino terminus and ectodomain, and the gp41 transmembrane region (9, 16, 21, 22, 27, 31, 33, 37, 42, 60, 81).

Here we examine the phenotypes associated with deletion of the major variable regions of the HIV-1 gp120 glycoprotein.

MATERIALS AND METHODS

Construction of mutated *env* genes. The mutants were constructed in the HXBc2 strain of HIV-1 by site-directed mutagenesis as previously described and cloned into the expressor plasmid pSVIIIenv (31, 59). The ΔV1/2 mutant contains a deletion of amino acids 121 to 203, with the sequence Gly-Ala-Gly replacing the missing sequences. (In this numbering system, 1 represents the initiator methionine.) The ΔV3 mutant, previously designated Δ297-329 (85), contains a deletion of amino acids 297 to 329 with a Gly-Ala-

* Corresponding author.

Gly sequence replacing the deleted V3 loop. In both deletions, the cysteines involved in disulfide bonding (at residues 119 and 205 for the V1/V2 loop and residues 295 and 331 for the V3 loop) are retained (45). The ΔV1/2/3 mutant contains both deletions and substitutions associated with the ΔV1/2 and ΔV3 mutants. The ΔV4 mutant contains a deletion of residues 396 to 413, which are replaced by the sequence Gly-Gly-Gly-Ala-Gly-Gly-Gly. The pSVIIIenvΔKS plasmid, which contains an out-of-frame deletion within the *env* gene, was used as a negative control for all the studies (77).

Expression of mutant envelope glycoproteins. COS-1 cells were transfected with the expressor plasmid DNA by the DEAE-dextran technique as described previously (59). Forty-eight hours following transfection, the cells were labeled in cysteine-free medium with 10% heat-inactivated fetal calf serum (FCS) and 40 μCi of [^{35}S]cysteine (Dupont, NEN Research Products) per ml for 16 h. Cells were washed with phosphate-buffered saline (PBS) and lysed in either radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) or NP40 buffer (0.5% Nonidet P-40, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5). Envelope glycoproteins were precipitated from the lysates with either a mixture of sera from AIDS patients or monoclonal antibodies as described previously (85). Cell supernatants were precipitated with the same antibodies and washed in RIPA buffer minus deoxycholate.

Cell surface expression of mutant glycoproteins. Sixty hours following transfection, the COS-1 cells were ^{125}I cell surface labeled by the lactoperoxidase method as previously described (85). The labeled cells were then lysed in RIPA buffer, immunoprecipitated with the AIDS patient sera, and analyzed by SDS gel electrophoresis.

Antibody binding to mutant glycoproteins expressed on the COS-1 cells. Binding of the F105, 1.5e, or 2.1h antibodies (35, 63, 76) to the mutant glycoproteins expressed on the COS-1 cells was assessed as described previously (9, 85). Briefly, ^{35}S-labeled monoclonal antibody was incubated with transfected COS-1 cells for 90 min at 37°C in medium containing 10% fetal calf serum and lysed in either RIPA or NP40 buffer. Bound antibody was precipitated by protein A-Sepharose (Pharmacia) and analyzed by SDS-polyacrylamide gel electrophoresis.

For measurements of F105 antibody affinity, the wild-type and ΔV1/2/3 mutant glycoproteins were expressed in COS-1 cells with the pcDNA1neo vector (Invitrogen), in which the *rev* and *env* genes were expressed under the control of the cytomegalovirus immediate-early promoter. Nearly confluent COS-1 cells were transfected with 10 μg of either the pcDNA1neo plasmid or the pcDNA1neo vector containing the wild-type or ΔV1/2/3 *env* genes, using the DEAE-dextran technique. Twenty-four hours later, the cells were split 1:2 into 100-mm petri dishes. At 48 h after transfection, the cells were labeled overnight with 200 μCi per plate of [^{35}S]cysteine in 5 ml of cysteine-free RPMI 1640 medium containing 10% heat-inactivated FCS. Following labeling, the medium was removed and the cells were washed once with PBS containing 2% FCS. The F105 antibody was serially diluted from 100 μg to 32 ng in 1 ml of RPMI 1640–10% FCS and added to the labeled cells for 90 min at 37°C with occasional gentle shaking. The cells were then washed three times with 5 ml of ice-cold PBS–2% FCS and lysed in NP40 buffer. The F105 antibody precipitates the wild-type and ΔV1/2/3 glycoproteins with equal efficiency from cell lysates prepared in NP40 buffer (see Results). Complexes of the HIV-1 envelope glycoproteins with F105 antibody were precipitated with protein A-Sepharose overnight at 4°C, washed three times with NP40 buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. To assess the background binding of the F105 antibody to COS-1 cells, unlabeled COS-1 plates transfected with the pcDNA1neo plasmid alone were treated with antibody, washed, and lysed as described above. These unlabeled lysates were transferred to radiolabeled plates containing COS-1 cells expressing the wild-type HIV-1 envelope glycoproteins, which were lysed and precipitated to assess nonspecific F105 antibody binding to control COS-1 cells. Values for both specific and nonspecific F105 binding were densitometrically determined from autoradiograms. Values for nonspecific F105 binding were negligible at antibody concentrations less than or equal to the saturating level observed for specific binding.

CD4 binding ability of mutant glycoproteins. The ability of the monomeric exterior envelope glycoproteins to bind soluble CD4 was assessed by incubating [^{35}S]cysteine-labeled supernatants from transfected COS-1 cells with increasing amounts of soluble CD4 (American BioTechnologies, Inc.) for 90 min at either 4 or 37°C. The envelope glycoproteins bound to soluble CD4 were precipitated with the OKT4 monoclonal antibody (Ortho Diagnostics) bound to protein A-Sepharose, using either 4 or 37°C overnight incubations. Unbound envelope glycoproteins were precipitated by a mixture of sera from AIDS patients bound to protein A-Sepharose.

The ability of soluble CD4 to bind the multimeric envelope glycoprotein complex was assessed by incubating transfected COS-1 cells with labeled soluble CD4. Soluble CD4 was labeled by the Bolton-Hunter method to a specific activity of 1.3 Ci/mmol. Sixty hours following transfection, COS-1 cells were washed with PBS and then incubated in PBS containing 5×10^{-8} M ^{125}I-soluble CD4 and 2% FCS for 90 min at 37°C. Cells were washed three times in PBS containing 2% FCS, lysed in NP40 buffer, and precipitated with OKT4 antibody.

Soluble CD4-induced shedding of exterior envelope glycoproteins. Transfected COS-1 cells were labeled with [^{35}S]cysteine as described above and incubated with 0, 10, or 30 μg of soluble CD4 in 1 ml of medium with 2% FCS for 90 min at 37°C. The medium was then precipitated with a mixture of sera from AIDS patients. Precipitates were washed and analyzed as described above.

Functional activity of envelope glycoprotein mutants. The ability of envelope glycoproteins expressed in transfected COS-1 cells to form syncytia with SupT1 CD4-positive lymphocytes was measured as previously described (77). The ability of the envelope glycoproteins to complement the entry of the *env*-deleted provirus, HXBΔ*env*CAT, into Jurkat lymphocytes was assessed as described previously (31).

RESULTS

Expression of mutant glycoproteins in transfected cells. In this study, large deletions were introduced into the major variable gp120 regions V1/V2, V3, and V4. An attempt was made to retain the potential for disulfide bond formation at the base of each of the variable loops (45). These mutants are referred to as ΔV1/2, ΔV3, and ΔV4. Another mutant (ΔV1/2/3) containing deletions of the V1/V2 and V3 regions was also constructed. The short V5 gp120 region was not altered in this study; the effects of amino acid changes in the V5 region of the HIV-1 gp120 glycoprotein have previously been reported (59).

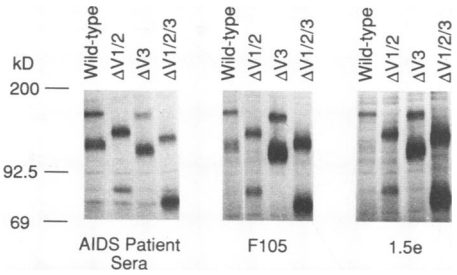

FIG. 1. Immunoprecipitation of the wild-type and deleted envelope glycoproteins in RIPA lysis buffer. Precipitation of the wild-type gp160 precursor (upper band) and processed gp120 glycoprotein (lower band) and of the precursor and processed forms of the mutant glycoproteins with a mixture of AIDS patient sera or the F105 or 1.5e monoclonal antibodies is shown.

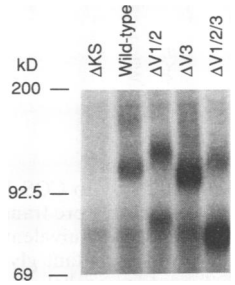

FIG. 2. Iodination of cell surface envelope glycoproteins. The wild-type and mutant envelope glycoproteins labeled by lactoperoxidase on the transfected COS-1 cell surface and precipitated with a mixture of sera from AIDS patients are shown. The identification of the observed precursor and processed glycoprotein bands can be made by reference to Fig. 1. Note that in the lane for the ΔV3 mutant, the faster-migrating band represents a background band, with the more intense, slowly migrating band representing the processed ΔV3 exterior envelope glycoprotein.

The mutant glycoproteins were expressed transiently in COS-1 cells as described previously (59, 77). The transfected cells were labeled and lysed in either RIPA or NP40 buffer (85). The HIV-1 envelope glycoproteins in the cell lysates were detected by precipitation with either a mixture of sera from AIDS patients or the F105 or 1.5e monoclonal antibodies. The F105 and 1.5e antibodies are neutralizing human monoclonal antibodies that are capable of blocking the gp120-CD4 interaction (35, 63). Figure 1 shows that the AIDS patient sera precipitated both precursor and processed forms of the wild-type and ΔV1/2, ΔV3, and ΔV1/2/3 glycoproteins from transfected COS-1 cells lysates. The ΔV3 glycoproteins were processed from the precursor more efficiently than the wild-type glycoprotein, whereas the ΔV1/2 mutant exhibited a decrease in precursor cleavage relative to the wild-type glycoprotein. The ΔV1/2/3 mutant was processed as efficiently as the wild-type glycoprotein. Only the precursor form of the ΔV4 mutant was precipitated from COS-1 cell lysates (data not shown). Both precursor and processed forms of the ΔV1/2, ΔV3, and ΔV1/2/3 mutants were precipitated by the F105 or 1.5e monoclonal antibodies from cell lysates. In cell lysates made in NP40 buffer, the mutant glycoproteins were precipitated by the F105 or 1.5e antibodies with an efficiency equivalent to those seen for the wild-type glycoproteins (data not shown). In cell lysates made in RIPA buffer, the F105 and 1.5e antibodies precipitated the ΔV3 and ΔV1/2/3 mutants significantly better than they precipitated the wild-type glycoproteins (Fig. 1). The increased ability to precipitate the ΔV3 and ΔV1/2/3 mutants in RIPA buffer was also observed for two other human monoclonal antibodies (2.1h and 1125H) directed against the gp120 CD4-binding region (79, 85; data not shown).

The amount of the mutant glycoproteins present in the supernatant of transfected COS-1 cells was examined by precipitation with a mixture of sera from AIDS patients. The level of processed exterior glycoprotein precipitated from supernatants of cells expressing the wild-type glycoproteins was greater than the levels seen for the ΔV1/2 or ΔV1/2/3 mutant. In contrast, approximately four- to fivefold more processed ΔV3 glycoprotein appeared in the supernatant than the amount observed for the wild-type glycoprotein (data not shown). No detectable ΔV4 glycoprotein was present in cell supernatants (data not shown). Apparently the deletion within the V4 region affects the proper processing of the HIV-1 envelope glycoprotein precursor.

Cell surface expression of HIV-1 envelope glycoprotein mutants. To estimate the level of cell surface expression of the mutant glycoproteins, transfected COS-1 cells were ^{125}I labeled by the lactoperoxidase method, lysed, and precipitated with a mixture of sera from AIDS patients. The wild-type gp120 glycoprotein was labeled by this procedure much more efficiently than was the gp160 precursor (Fig. 2), consistent with previous observations of transfected COS-1 cells (9, 85). The processed forms of the ΔV1/2, ΔV3, and ΔV1/2/3 mutant glycoproteins were efficiently labeled by lactoperoxidase. For the ΔV1/2 mutant, equal amounts of the precursor and processed forms of the glycoproteins were detected, whereas for the ΔV3 and ΔV1/2/3 mutants, little precursor protein was detected in this assay. When comparable amounts of plasmid DNA were used for the transfection, more of the ΔV1/2/3 processed exterior glycoprotein was detected on the cell surface than that observed for the wild-type glycoproteins. The ΔV1/2 and ΔV3 glycoproteins demonstrated only slight (four- and threefold, respectively) increases in total protein detected on the cell surface relative to the wild-type glycoproteins under these conditions.

Recognition of envelope glycoprotein complexes by neutralizing antibodies. To examine whether the mutant glycoproteins on the cell surface retained the ability to be recognized by an antibody that reacts with a conformation-sensitive epitope, the binding of ^{35}S-labeled F105 antibody to the transfected COS-1 cells was measured. For this experiment, equal amounts of plasmid DNA (10 μg) encoding the wild-type, ΔV1/2, and ΔV3 glycoproteins were transfected into COS-1 cells. A lower amount of plasmid DNA (20 ng) encoding the ΔV1/2/3 glycoprotein was transfected so that levels of cell surface expression of this mutant would approximate that of the other three glycoproteins. Half of the transfected COS-1 cells were used for surface iodination by lactoperoxidase, so that the level of cell surface envelope glycoprotein expression could be verified. Half of the transfected cells were incubated with ^{35}S-labeled F105 antibody. As shown in Fig. 3, little nonspecific binding of the labeled F105 antibody to the COS-1 cells transfected with the pSVIIIenvΔKS plasmid, which contains a large deletion in the *env* gene, was observed. The COS-1 cells expressing the wild-type and mutant glycoproteins bound significantly greater amounts of the labeled antibody. F105 antibody binding to the COS-1 cells expressing the ΔV1/2 and ΔV1/2/3 mutants was 2.4-fold and 13-fold, respectively, greater than that observed for cells expressing the wild-type glycoproteins when the amount of glycoprotein expression on the cell

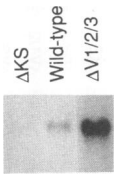

FIG. 3. Binding of F105 antibody to COS-1 cells expressing the envelope glycoproteins. COS-1 cells were transfected with amounts of plasmid DNA adjusted to obtain equivalent cell surface expression of the wild-type and ΔV1/2/3 mutant glycoproteins, as determined by iodination conducted in parallel. Labeled F105 antibody was bound to the cells, which were lysed and incubated with protein A-Sepharose. The bound F105 heavy-chain band, resolved by SDS-PAGE, is shown.

surface was normalized (data not shown and Fig. 3). Increases in binding of two other labeled antibodies directed against the gp120 CD4 binding site (1.5e and 2.1h) were also observed for the ΔV1/2 and ΔV1/2/3 mutants, relative to that seen for the wild-type glycoproteins (data not shown).

The above studies suggested that, even when the amount of lactoperoxidase-labeled cell surface glycoproteins was normalized, antibodies directed against the CD4 binding region exhibited greater recognition of the ΔV1/2/3 mutant than the wild-type glycoproteins. Assuming that the iodination efficiencies of the ΔV1/2/3 mutant and wild-type glycoproteins were comparable, the results suggest the possibility that the monoclonal antibodies exhibit an increased affinity for the ΔV1/2/3 complex. To test this possibility, we directly measured the affinity of the F105 antibody for the wild-type and ΔV1/2/3 envelope glycoproteins on the surface of transfected COS-1 cells. Figure 4 shows that half-saturating concentrations of F105 antibody, which indicate the antibody dissociation constants, for the wild-type and ΔV1/2/3 glycoproteins were 133 and 2.7 nM, respectively. We conclude that deletion of the ΔV1/2 and V3 regions results in a

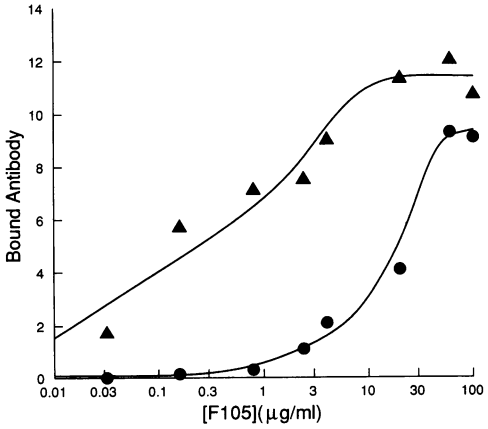

FIG. 4. Measurement of F105 antibody binding to wild-type and ΔV1/2/3 envelope glycoproteins expressed on the surface of COS-1 cells. COS-1 cells were transfected with equal amounts of plasmid DNA expressing either the wild-type (●) or ΔV1/2/3 (▲) envelope glycoproteins. The transfected cells were incubated with serial dilutions of the F105 antibody, and the amount of bound antibody was determined as described in Materials and Methods. The amount of bound antibody is expressed in arbitrary densitometric units. The half-saturating concentrations of F105 antibody were 133 and 2.7 nM, respectively, for the wild-type and ΔV1/2/3 glycoproteins.

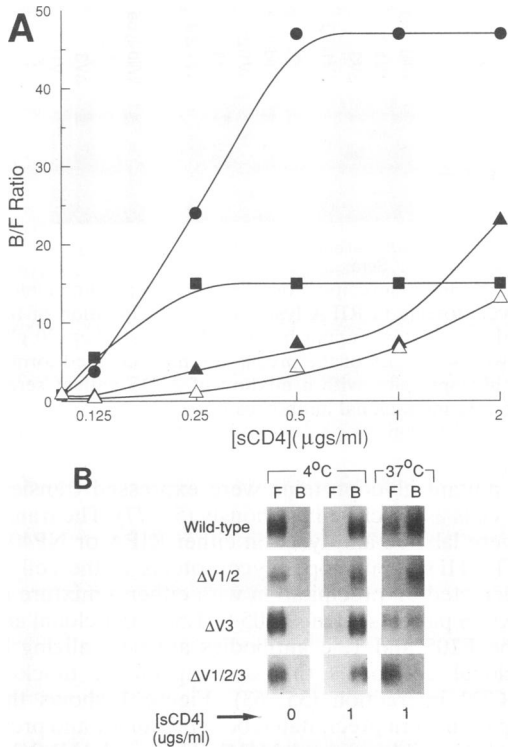

FIG. 5. Binding of soluble forms of the exterior glycoprotein to soluble CD4 at 4 and 37°C. (A) Binding of soluble wild-type (●), ΔV1/2 (■), ΔV3 (△), and ΔV1/2/3 (▲) exterior glycoproteins from COS-1 supernatants to soluble CD4 (sCD4) at 4°C. Values shown represent the ratio of soluble CD4-bound gp120/freegp120 (B/F). The B/F ratios shown for the wild-type and ΔV1/2 envelope glycoproteins at saturating concentrations of soluble CD4 represent minimal estimates of the true B/F ratio, since the amount of free glycoprotein was below detectable limits. (B) Binding of soluble wild-type and mutant exterior glycoproteins to soluble CD4 at 4 and 37°C. The amount of soluble glycoproteins free (F) or bound (B) to 1 μg of soluble CD4 (sCD4) per ml is shown. The pattern of background binding in the absence of soluble CD4 was identical at both temperatures, so only the 4°C data are shown.

significant increase in the affinity of the F105 antibody for the envelope glycoprotein complex on the cell surface.

CD4 binding ability of envelope glycoprotein mutants. The ability of the deletion mutants to bind CD4 was assessed in two ways. In the first assay, radiolabeled exterior glycoproteins in the COS-1 cell supernatants were incubated with increasing concentrations of soluble CD4 (4, 15, 20, 36, 71, 80). The amount of envelope glycoprotein bound to the soluble CD4 was estimated by precipitation of the envelope glycoprotein-soluble CD4 complexes by the OKT4 monoclonal antibody (49). The unbound envelope glycoproteins were precipitated by sera from AIDS patients. The results are shown in Fig. 5. Although the differing amounts of the wild-type and ΔV1/2 glycoproteins present in the cell supernatants resulted in different estimates of saturating bound/free ratios, the half-saturating soluble CD4 concentrations, reflecting the dissociation constants, indicated a slightly higher affinity for the ΔV1/2 glycoprotein relative to that of the wild-type gp120 glycoprotein at 4°C. By contrast, the 4°C binding of the ΔV3 and ΔV1/2/3 glycoproteins was less efficient than that of the wild-type glycoprotein. At 37°C, the wild-type and mutant glycoproteins bound soluble CD4 less

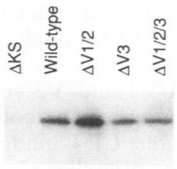

FIG. 6. Binding of ^{125}I-labeled soluble CD4 to the surfaces of COS-1 transfectants expressing wild-type and mutant envelope glycoproteins. Following a 90-min incubation at 37°C, bound soluble CD4 was analyzed by precipitation with OKT4 antibody and SDS-PAGE.

efficiently than at 4°C (Fig. 5B). At 37°C, as at 4°C, both the ΔV3 and the ΔV1/2/3 mutants exhibited decreases in soluble CD4 binding relative to the wild-type glycoprotein, whereas the binding of the ΔV1/2 glycoprotein was better than that of the wild-type glycoprotein.

In the second assay, the ability of the multimeric envelope glycoprotein complexes to bind CD4 was examined (Fig. 6). COS-1 cells were transfected with amounts of plasmid DNA as described above for the antibody-binding studies. Labeled soluble CD4 was incubated at 37°C with transfected COS-1 cells, which were then washed, lysed, and precipitated with OKT4 antibody. At the concentrations of labeled soluble CD4 used in this assay, shedding of the exterior glycoprotein is minimal (54, and see below). Negligible binding to COS-1 cells transfected with the pSVIIIenvΔKS control plasmid was observed. When the amounts of cell surface expression of the envelope glycoprotein mutants were taken into account, the ΔV1/2, ΔV3, and ΔV1/2/3 glycoproteins bound soluble CD4 with efficiencies, relative to that of the wild-type glycoproteins, of 0.6, 0.3, and 0.25, respectively.

Dissociation of exterior glycoprotein by soluble CD4. High concentrations of soluble CD4 induce the shedding of the wild-type HXBc2 gp120 glycoprotein from the multimeric envelope glycoprotein complex expressed on the surface of COS-1 cells (5, 8, 54). To examine whether soluble CD4 would exert a similar effect on the mutant glycoproteins, we metabolically labeled transfected COS-1 cells with [^{35}S]cysteine and incubated them with increasing amounts of soluble CD4 at 37°C. The COS-1 supernatants were precipitated with a mixture of sera from AIDS patients. As shown in Fig. 7, increasing amounts of the wild-type gp120 glycoprotein were evident in COS-1 supernatants following incubation with soluble CD4. Comparable increases in the amounts of exterior glycoprotein shed into the supernatant were seen for the ΔV3 mutant. Neither the ΔV1/2 nor the ΔV1/2/3 glycoproteins exhibited increases in the amount of the exterior envelope glycoprotein appearing in the cell supernatants following soluble CD4 treatment.

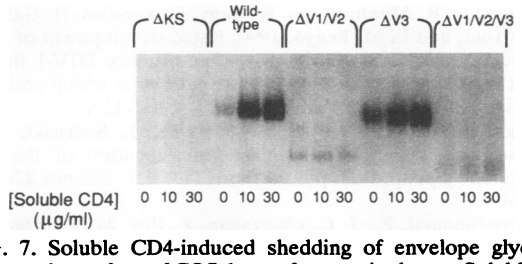

FIG. 7. Soluble CD4-induced shedding of envelope glycoproteins from the surface of COS-1 transfectants is shown. Soluble CD4 was incubated with the envelope glycoprotein-expressing cells for 90 min at 37°C and soluble CD4-induced shedding of gp120 was analyzed by immunoprecipitation of cell supernatants with a mixture of sera from AIDS patients.

Syncytium-forming and replicative ability of mutant glycoproteins. To examine the functional capability of the mutant glycoproteins, we cocultivated transfected COS-1 cells with CD4-positive SupT1 lymphocytes. Syncytia were counted following an overnight incubation. None of the deletion mutants induced the formation of syncytia at a level greater than did the pSVIIIenvΔKS negative control. The wild-type glycoproteins under these conditions formed over 400 syncytia per ml (data not shown). Similarly, the deletion mutants did not complement the ability of an *env*-defective HIV-1 provirus to enter Jurkat lymphocytes (data not shown). Thus, the deletion mutants appear to be defective for both syncytium-forming and replicative ability.

DISCUSSION

The effect of deletions involving the major variable regions of the gp120 glycoprotein on structure and function of the HIV-1 envelope glycoproteins was investigated. Major deletions in the V1/V2 and/or V3 regions did not abrogate the processing or transport to the cell surface of the envelope glycoproteins, suggesting that proper folding of the HIV-1 envelope glycoprotein can occur in the absence of these regions. That these glycoproteins bind soluble CD4, the gp41 glycoprotein, and conformation-dependent monoclonal antibodies also supports the notion that a structure resembling that present on the native gp120 glycoprotein can be achieved by these mutants. The presence of the uncleaved ΔV1/2 precusor on the cell surface indicates that the slight decrease in precursor processing observed for this mutant was not accompanied by retention in the endoplasmic reticulum, a fate associated with misfolded envelope glycoproteins (9, 17, 18). Thus, inhibition of cleavage per se rather than misfolding probably accounts for this observation. The decrease in precusor processing observed for the ΔV1/2 mutant was apparently compensated by the deletion of the V3 region in the ΔV1/2/3 mutant. Deletion of the V3 region alone also accelerated the rate of processing of the ΔV3 glycoprotein. These results suggest that elements of the V3 loop may mask the gp120-gp41 cleavage site and that this masking may be accentuated by deletion of the V1/2 structure. An increased steady-state level of cell surface expression, in the absence of an increase in overall expression, was observed for the ΔV1/2/3 mutant relative to that seen for the wild-type glycoproteins. This indicates either that the processing and transport of this mutant glycoprotein to the cell surface is accelerated or that the cell surface mutant glycoprotein is more stable than the wild-type glycoprotein. Partial deletion of the V4 region apparently interferes with the proper folding of the precursor glycoprotein, since cleavage into mature envelope glycoproteins was completely abrogated. Nonprocessed HIV-1 envelope glycoproteins were observed in another study in which a different V4 deletion was created (62).

The efficient expression of the ΔV1/2/3 exterior envelope glycoprotein on the surface of transfected COS-1 cells clearly demonstrates that sequences in V1, V2, or V3 are not required for the association of the gp120 glycoprotein with the gp41 transmembrane glycoprotein. Slight increases in the amount of the exterior envelope glycoprotein present in the transfected cell supernatants were observed for the ΔV3 mutant, relative to that seen for the wild-type glycoproteins. This observation is consistent with the mild increase in cell

surface expression of this mutant glycoprotein and perhaps a mild decrease in subunit association. Amino acid changes in the V3 loop have in some cases resulted in decreases in gp120-gp41 association, especially for HIV-1 envelope glycoproteins made in lymphocytes (42). The reported proximity of the V3 loop to the fourth conserved gp120 region (85), changes in which can decrease gp120-gp41 affinity (32), may account for some of these effects. The levels of the ΔV1/2 and ΔV1/2/3 glycoproteins in transfected cell supernatants were less than those of the wild-type glycoproteins despite increased relative cell surface expression of these mutants. This suggests that the association of these mutant exterior glycoproteins with the gp41 glycoprotein may be more stable than that of the wild-type glycoprotein.

The mutant glycoproteins containing large deletions in V1/V2 and V3 retained the ability to bind soluble CD4, both as monomers and as multimeric complexes. Efficient CD4 binding has been reported previously for HIV-1 gp120 glycoproteins with deletions involving these variable regions (59, 62, 81). Our results indicate that deletion of the V3 loop, but not the V1/V2 region, is associated with a decrease in CD4 binding affinity. Previous studies suggesting that HIV-1 gp120 glycoproteins containing deletions in the V3 loop efficiently bound CD4 were not designed to provide a quantitative estimate of binding affinity (62, 81).

Dissociation of the exterior envelope glycoprotein from the gp41 glycoprotein by soluble CD4 occurs at 37°C for some HIV-1 isolates, including the HXBc2 strain utilized in this study (30, 43, 53, 54). Extensive mutagenic studies indicate that a high affinity for CD4 is necessary but not sufficient for soluble CD4-induced shedding of the gp120 glycoprotein and that the shedding of gp120 is not necessarily predictive of the membrane fusion capacity of a given envelope glycoprotein (6, 13, 53, 75). Deletion of the V3 loop did not affect the efficiency of soluble CD4-induced shedding of the exterior glycoprotein. By contrast, neither of the mutants containing deletions of the V1/V2 region exhibited substantial shedding of the exterior envelope protein following soluble CD4 treatment, although these mutants were capable of binding soluble CD4 under these conditions. These results suggest that the deletion of the V1/V2 region affects the shedding process per se. While it is clear that the V1/V2 region is not necessary for gp120 association with the gp41 glycoprotein, changes in the V1/V2 structure can result in dissociation of the HIV-1 envelope glycoprotein subunits (42, 74a). Thus, it is possible that soluble CD4 binding to the wild-type glycoprotein complex can elicit changes in the structure of the V1/V2 region that result in a decrease in subunit affinity. In this study, the ΔV1/2 and ΔV3 mutants differed dramatically in shedding of the exterior glycoprotein in response to soluble CD4, yet both are defective for membrane fusion. These results are consistent with previous studies reporting that soluble CD4-induced shedding and conformational changes in the HIV-1 envelope glycoproteins relevant to the membrane fusion process do not appear to correlate (6, 13, 53, 69, 75).

The ΔV1/2 mutant is expressed on the transfected cell surface, binds CD4, but does not induce the formation of syncytia or mediate virus entry. Thus, this mutant appears to be defective for some aspect of the membrane fusion process. Single amino acid changes, deletions, or insertions affecting the V3 loop have previously been shown to disrupt the fusion process (22, 27, 31, 37, 60, 81). These studies suggest that particular elements of the V1/V2 region may also play a role in governing the efficiency of induction of membrane fusion. This is consistent with the observation that some antibodies directed against sequences in the V2 region do not block gp120-CD4 binding yet neutralize HIV-1 (23, 34).

Removal of the V1/V2 and V3 regions can be accomplished without disruption of the conserved discontinuous epitopes overlapping the CD4 binding site that serve as targets for neutralizing antibodies (7, 11, 28, 35, 40, 63, 73, 79). Under some buffer conditions, deletion of the V3 loop alone enhances the exposure of epitopes that include components derived from the fourth conserved (C4) region of the HIV-1 gp120 glycoprotein, consistent with previously reported results (85). Under native conditions, removal of the variable regions V1/V2 and V3 enhances the accessibility of several neutralizing anti-CD4 binding antibodies to the multimeric envelope glycoprotein complex. Thus, the exposure of a major group of conserved neutralization epitopes on the HIV-1 envelope glycoprotein complex is apparently masked by the presence of surface-accessible loop structures containing variable neutralization epitopes. These structural features can explain the more rapid appearance of neutralizing antibodies recognizing the gp120 variable loops relative to those recognizing more conserved epitopes in HIV-1-infected chimpanzees (1, 7, 10, 11, 25, 26, 35, 52, 56, 57, 64, 66, 82–84, 86). During the early months of infection, the humoral antiviral response is thus directed at envelope glycoprotein regions that can be readily altered with little compromise to viral infectivity. HIV-1 may be less capable of dealing with antibodies directed against the more conserved gp120 regions during the establishment phase of infection, underscoring the potential benefit of these antibodies in prophylaxis. Unfortunately, the elicitation of broadly neutralizing antibodies directed against conserved gp120 regions has proven difficult (29, 51, 61), an observation possibly related to the masking phenomenon described above. Thus, deleted versions of the HIV-1 envelope glycoproteins modified to optimize the exposure of conserved, conformation-dependent neutralization targets may prove useful in eliciting effective prophylactic immune responses.

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