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Involvement of the V1/V2 Variable Loop Structure in the Exposure of Human Immunodeficiency Virus Type 1 gp120 Epitopes Induced by Receptor Binding

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The binding of human immunodeficiency virus type 1 (HIV-1) to the cellular receptor CD4 has been suggested to induce conformational changes in the viral envelope glycoproteins that promote virus entry. Conserved, discontinuous epitopes on the HIV-1 gp120 glycoprotein recognized by the 17b, 48d, and A32 antibodies are preferentially exposed upon the binding of soluble CD4 (sCD4). The binding of the 17b and 48d antibodies to the gp120 glycoprotein can also be enhanced by the binding of the A32 antibody. Here we constructed HIV-1 gp120 mutants in which the variable segments of the V1/V2 and V3 structures were deleted, individually or in combination, while the 17b, 48d, and A32 epitopes were retained. The effects of the variable loop deletions on the function of the HIV-1 envelope glycoproteins and on the exposure of epitopes induced by sCD4 or A32 binding to the monomeric gp120 glycoprotein were examined. The variable-loop-deleted envelope glycoproteins were able to mediate virus entry, albeit at lower efficiencies than those of the wild-type glycoproteins. Thus, the V1/V2 and V3 variable sequences contribute to the efficiency of HIV-1 entry but are not absolutely required for the process. Neither the V1/V2 nor V3 loops were necessary for the increase in exposure of the 17b/48d epitopes induced by binding of the A32 monoclonal antibody. By contrast, induction of the 17b, 48d, and A32 epitopes by sCD4 binding apparently involves a movement of the V1/V2 loops, which in the absence of CD4 partially mask these epitopes on the native gp120 monomer. The results obtained with a mutant glycoprotein containing a deletion of the V1 loop alone indicated that the contribution of the V2 loop to these phenomena was more significant than that of the V1 sequences. These results suggest that the V1/V2 loops, which have been previously implicated in CD4-modulated, postattachment steps in HIV-1 entry, contribute to CD4-induced gp120 conformational changes detected by the 17b, 48d, and A32 antibodies.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (3, 27). The entry of HIV-1 into target cells, like that of other retroviruses, is mediated by the viral envelope glycoproteins. The gp120 exterior envelope glycoprotein binds to the CD4 receptor, while the gp41 transmembrane envelope glycoprotein serves to anchor the tetrameric envelope glycoprotein complex in the viral membrane (1, 18, 41, 46, 48, 63). Following receptor binding, the envelope glycoproteins mediate the fusion of viral and target cell membranes, which is critical for successful entry of the viral core into the cell (73).

Most of the surface-exposed elements of the mature envelope glycoprotein complex are contained on the gp120 exterior envelope glycoprotein (53, 67). When the gp120 glycoproteins derived from different HIV-1 isolates are compared, five conserved regions (C1 to C5) and five variable regions (V1 to V5) can be identified (57, 71). Efficient CD4 binding is dependent on discontinuous elements derived from the third (aspartic acid 368 and glutamic acid 370) and fourth (tryptophan 427 and aspartic acid 457) conserved regions (15, 42, 44, 58). Conserved, discontinuous epitopes overlapping the CD4 binding site serve as targets for neutralizing antibodies (34, 40, 61, 72, 78, 81, 82).

Intramolecular disulfide bonds in the gp120 glycoprotein result in the inclusion of the first four variable regions (V1 to V4) in large loop-like structures (45). Antibody mapping studies indicate that, of the linear epitopes on the gp120 glycoprotein, those located in the V2 and V3 regions constitute the most exposed elements on the HIV-1 multimeric envelope glycoprotein complex (53). Both V2 and V3 loops can serve as targets for neutralizing antibodies (26, 33, 38, 49, 54, 62, 64, 69). Changes in V2 and V3 loop amino acids have been shown to affect the membrane fusion process and can influence the tropism of HIV-1 isolates for primary macrophages and T-lymphocyte lines (4, 7, 10–12, 24, 28, 29, 31, 36, 37, 59, 68, 75, 84, 85). Amino acid alterations in the fourth conserved (C4) region, which has been suggested to interact with the V3 loop (55, 56, 76, 89), can also affect membrane fusion events involved in HIV-1 entry (77).

Analogy with the influenza virus hemagglutinin (86) suggests that, after receptor binding, conformational changes in the HIV-1 envelope glycoproteins may have to occur to allow exposure of the gp41 glycoprotein to the target cell membrane. Mutagenic analysis has defined gp41 regions important for membrane fusion, and the locations of these regions are consistent with models proposed for the orthomyxoviruses (8, 20, 23, 32, 42). Unlike the orthomyxoviruses, however, HIV-1 does

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not require a decrease in pH to trigger the membrane fusion process (73). Instead, it has been suggested that CD4 binding serves as a trigger for fusion-related conformational changes. Such a model is consistent with the observation that a soluble form of the CD4 receptor (sCD4) (19, 21, 35, 70, 83) can enhance the infection of HIV-2 (14), simian immunodeficiency virus (SIVagm) (2), and some isolates of HIV-1 (74).

The binding of sCD4 has been shown to induce conformational changes in the HIV-1 envelope glycoproteins. For T-cell-line-passaged HIV-1 isolates, sCD4 binding results in the shedding of the gp120 glycoprotein from the envelope complex (6, 30, 50). This observation, combined with the report that the HIV-1 gp41 glycoprotein alone could mediate fusion (60), suggested a model in which CD4 binding exposed the gp41 glycoprotein by removing the gp120 glycoprotein from the envelope glycoprotein complex. More recent data, however, have cast doubt on both the relevance of gp120 shedding to the membrane fusion process (4, 17, 25, 51, 52, 77, 80) and on the reproducibility of cell fusion mediated by the isolated gp41 glycoprotein (47).

The uncertain relevance of gp120 shedding to HIV-1 entry has prompted a search for other markers of fusion-related conformational changes. A recent analysis of the effects of CD4 amino acid changes on HIV-1 entry suggests that conformational changes within the gp120-CD4 binding site itself are unlikely to be required for promoting subsequent fusion-related events in virus entry (5). Instead, regions of the gp120 glycoprotein outside the CD4 binding site, some of which have been reported to undergo conformational changes upon CD4 binding (39, 65, 66, 79), might be better candidates for structures involved in conformational changes related to virus entry. CD4 binding has been reported to increase the accessibility of the V3 loop to proteases, whereas the binding of some neutralizing antibodies to the V2 loop is decreased upon CD4 binding (49, 65, 66, 69). Discontinuous epitopes recognized by the 17b and 48d neutralizing antibodies are more exposed after CD4 binding (79). Mutagenic analysis of the HIV-1 gp120 glycoprotein has suggested that the conserved 17b and 48d epitopes are dependent upon the stem of the V1/V2 stem-loop structure, the fourth (C4) conserved region, and amino acids near or within the CD4 binding site (79).

A recent study of HIV-1 variants adapted to replicate on CD4 mutants with lower gp120 binding affinities suggested that the fusion-related functions of the V2, V3, and C4 regions of the gp120 glycoprotein could be modulated by CD4 binding (13). The involvement of the gp120 V2 and V3 loops in fusion events specifically modulated by CD4 binding prompted us to examine whether these variable structures might be involved in some of the conformational changes in the HIV-1 gp120 glycoprotein induced by receptor binding. The 17b and 48d neutralization epitopes, which are more exposed on both monomeric gp120 and oligomeric envelope glycoproteins of HIV-1 following sCD4 binding (79), were studied. The A32 human monoclonal antibody was also included in this study, because its binding to the monomeric gp120 glycoprotein is also enhanced by sCD4 binding and because A32 antibody binding to the gp120 glycoprotein results in enhanced binding of the 17b and 48d antibodies (63a).

MATERIALS AND METHODS

Construction of mutated *env* genes. The mutants were constructed in the HXBc2 strain of HIV-1 (22) by site-directed mutagenesis as previously described (43, 58) and cloned into the expressor plasmid pSVIIIenv. The Δ136-151 mutant contains a deletion of residues 136 to 151, with the sequence Gly-Ala replacing the missing sequences (see Fig. 1). (In the numbering system used, 1 represents the initiator methionine.) The Δ128-194 mutant contains a deletion of amino acids 128 to 194, with a Gly-Ala-Gly sequence replacing the missing V1/V2 loops. The Δ303-323 mutant contains a deletion of residues 303 to 323, with a Gly-Ala sequence replacing the missing V3 loop amino acids. The Δ128-194/303-323 mutant contains both deletions and substitutions associated with the individual Δ128-194 and Δ303-323 mutants. The ΔV1/V2 and ΔV3 mutants, which contain more-extensive deletions of the respective variable loop structures, were previously characterized (88) and are herein designated Δ121-203 and Δ298-329, respectively. The pSVIIIenvΔKS plasmid, which contains an out-of-frame deletion within the *env* gene, was used as a negative control for some of the experiments.

Expression of mutant envelope glycoproteins. COS-1 cells were transfected with the expressor plasmid DNA by the DEAE-dextran technique as described previously (16). Forty-eight hours following transfection, the cells were labeled in cysteine-free medium with 10% heat-inactivated fetal calf serum (HIFCS) 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 Nonidet P-40 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 (1 μg/ml final concentration) as described previously (58, 81). Cell supernatants were precipitated with the same antibodies (0.66 μg/ml) in the absence of detergent and washed with PBS and 2% HIFCS. sCD4 (Agmed) (10 μg/ml) was added to some of the cell lysates and supernatants prior to immunoprecipitation. Precipitates were analyzed on sodium dodecyl sulfate-polyacrylamide gels.

ELISA determination of mutant gp120 recognition by antibodies. COS-1 cells were transfected with 10 μg of pSVIIIenv DNA expressing wild-type or mutant HXBc2 envelope glycoproteins and a Tat-expressing plasmid, pSVTat. Seventy-two hours after transfection, cell supernatants were collected and frozen. For analysis of antibody recognition, various amounts of the supernatants (1 to 100 μl), supplemented with Tris-buffered saline–10% HIFCS to a total volume of 100 μl, were incubated in wells of Immulon II enzyme-linked immunosorbent assay (ELISA) plates (Dynatech, Ltd.) coated with sheep antibody D7324 to the carboxyl-terminal 15 amino acids of gp120. The amount of each mutant glycoprotein captured was estimated with the 133/290 monoclonal antibody (57a) directed against C1 gp120 residues 61 to 70. On the basis of this determination, the volume of each supernatant added to the ELISA plate was adjusted, so that equal amounts of each mutant glycoprotein were captured. The accuracy of this adjustment was verified by examining the amount of bound 133/290 antibody over a range (0.03 to 10 μg/ml) of antibody concentrations. For all experiments, 133/290 antibody was diluted in TMSS buffer (Tris-buffered saline containing 2% nonfat milk powder, 20% sheep serum) and reacted with the captured gp120 glycoprotein, and bound antibody was detected with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Accurate Chemicals) and the AMPAK system (DaKo Diagnostics).

The CD4 binding abilities of the captured mutant glycoproteins were determined by incubating CD4-immunoglobulin G (IgG) (Genentech) (9) diluted in TMSS buffer at various concentrations (0.02 to 6 μg/ml) with the captured gp120 glycoprotein, followed by detection with alkaline phosphatase-conjugated goat anti-human immunoglobulin (Accurate Chemicals) and the AMPAK system.

The binding of the 17b, 48d, and A32 antibodies in the absence or presence of 5 μg of sCD4 per ml was determined as described above for the binding of CD4-IgG. The 17b and 48d antibodies were tested over a concentration range of 0.01 to 3 μg/ml, while the A32 antibody was tested over a range of 0.03 to 10 μg/ml.

The effects of binding the A32 antibody to the captured gp120 glycoprotein on the binding of 17b and 48d antibodies were determined with biotinylated 17b and 48d antibodies in the absence or presence of 3 μg of the A32 antibody per ml. The bound biotinylated antibody was detected with streptavidin-conjugated alkaline phosphatase and the AMPAK system.

For some of the experiments, the envelope glycoproteins were captured on ELISA plates with the 133/290 antibody. The 133/290 antibody was coated onto the ELISA plates at 20 μg/ml, and the amount of captured envelope glycoprotein was normalized by using biotinylated 21h antibody, which recognizes an epitope overlapping the CD4 binding site (78). The binding of biotinylated 17b and 48d antibodies to the captured envelope glycoproteins in the absence or presence of 5 μg of sCD4 per ml was monitored with alkaline phosphatase-conjugated streptavidin and the AMPAK system described above.

All ELISA datum points shown in Fig. 3 to 5 represent the means of duplicate determinations; in a typical experiment, variation was less than 15% of the mean optical density value. Experiments were repeated at least twice with comparable results.

sCD4-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 sCD4 in 1 ml of medium with 2% HIFCS 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 activities of envelope glycoprotein mutants. The abilities of envelope glycoproteins expressed in transfected COS-1 cells to induce the formation of syncytia following cocultivation with SupT1 CD4-positive lymphocytes were measured as follows. For each envelope construct, a 100-mm-diameter dish of subconfluent COS-1 cells was transfected with 10 μg of plasmid DNA by the DEAE-dextran method as previously described (80). The next day, the cells were

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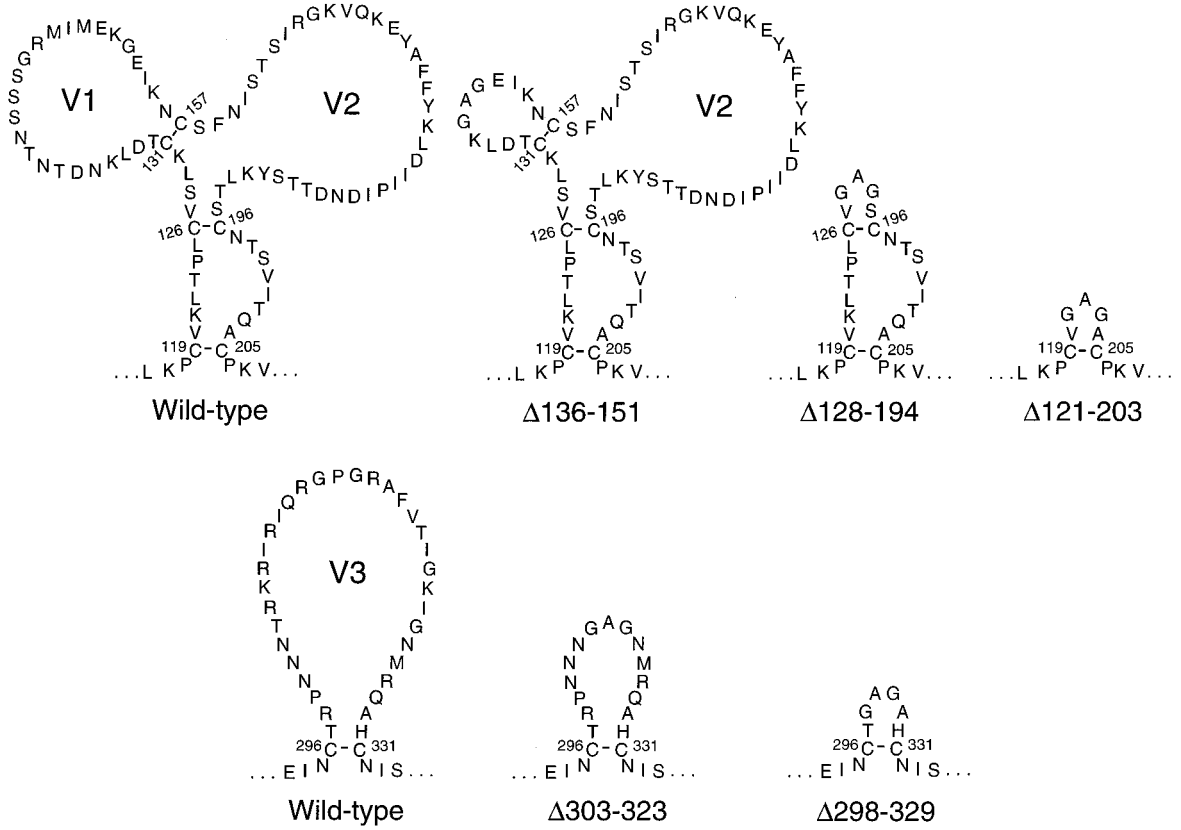

FIG. 1. Mutant envelope glycoproteins. The predicted sequence of each of the mutant glycoproteins in the region of the deletion is shown. The amino acid numbers corresponding to the cysteine residues are shown.

detached from the plate by incubation with trypsin-EDTA. The COS-1 cells were suspended in a volume of 12 ml of Dulbecco modified Eagle medium (DMEM) containing 10% HIFCS (DMEM+HIFCS). Two milliliters of the suspension was added to one well of a six-well tissue culture plate. Approximately 24 h later, SupT1 cells were suspended in DMEM+HIFCS at a concentration of 10^6/ml. The medium was removed from the COS-1 cells and replaced with 2 ml of the SupT1 cell suspension. The next day, trituration of the medium above the COS-1 monolayer in the cocultivation was performed to detach the syncytia. The syncytia and suspended SupT1 cells were diluted 1:10 in PBS, and 1 ml of the dilution mixture was added to a 24-well plate to score syncytia.

The abilities of the envelope glycoproteins to complement the entry of the *env*-deleted provirus HXBΔ*env*CAT into Jurkat lymphocytes were assessed as described previously (31, 80). The pSVIIIenvΔKS plasmid, which contains a deletion and frameshift mutation within the *env* gene, was used as a negative control in these experiments.

RESULTS

Effects of variable loop deletions on the HIV-1 envelope glycoproteins. To study the contribution of the V1/V2 and V3 variable loops to the induction of conformational changes in the HIV-1 gp120 glycoprotein by CD4 binding, gp120 mutants in which large portions of these regions were deleted but which retained the CD4-induced epitopes were constructed. Previous studies showed that deletion of the complete V1/V2 stem-loop structure disrupted the 17b and 48d epitopes and that recognition of these mutant glycoproteins was not restored by CD4 binding (79). Other data suggested that the conserved stem of the V1/V2 structure might contribute to the 17b and 48d epitopes (79). Complete deletion of the V3 loop also disrupted the binding of 17b and 48d antibodies, but recognition of this mutant could be restored by sCD4 binding (79). These results suggested that the deletion of the V3 loop disrupted local gp120 conformation and thus compromised the integrity of the 17b and 48d epitopes but that V3 sequences did not directly contribute to the formation of either epitope. Thus, new gp120 mutants in which conserved regions in the stem of the V1/V2 structure or at the base of the V3 loop were preserved were created. These mutants are shown in Fig. 1 and are compared with the previously reported mutants. The Δ128-194 mutant contains a complete deletion of the V1/V2 variable structures but retains the conserved stem at the base of the V1/V2 variable loops. The Δ136-151 mutant contains a deletion affecting only the V1 loop. The Δ303-323 mutant contains a deletion of the variable tip of the V3 loop, retaining the more conserved residues near the base of the loop. The Δ128-194/303-323 mutant contains a combined deletion of the variable portions of the V1/V2 and V3 loops.

The wild-type and mutant glycoproteins were expressed transiently in COS-1 cells, which were radiolabeled and used for immunoprecipitation by either monoclonal antibodies or a mixture of sera from AIDS patients. Since the AIDS patient sera recognize a number of different epitopes, precipitation with the mixture of sera allows an assessment of the relative steady-state levels of the wild-type and mutant glycoproteins. Figure 2A shows that the processing of the precursor forms of most mutant envelope glycoproteins was comparable to that of the wild-type envelope glycoproteins. The complete deletion of the V1/V2 stem-loop structure (Δ121-203) resulted in a decrease in the efficiency of precursor processing, as previously reported (88). The processed mutant exterior envelope glycoproteins were observed in the COS-1 supernatants (Fig. 2B). Compared with the level for the wild-type gp120 glycoprotein,

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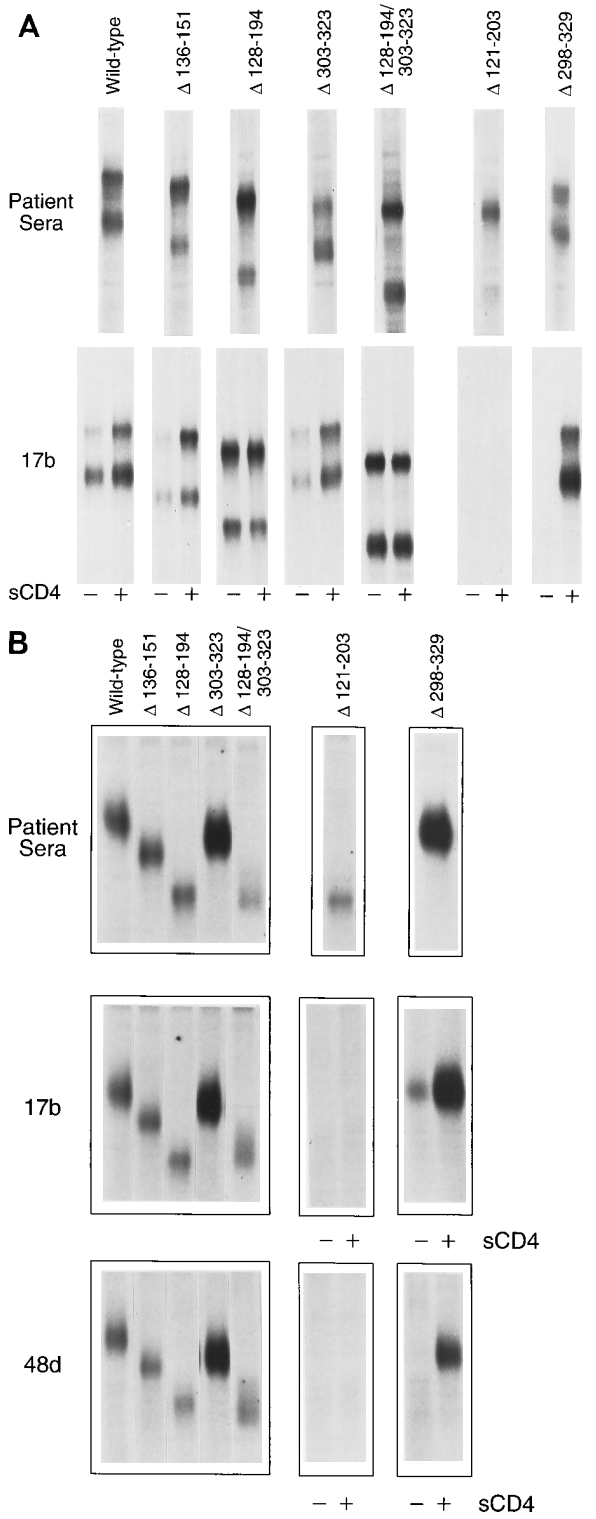

FIG. 2. Precipitation of wild-type and mutant glycoproteins. (A) Precipitation of labeled COS-1 cell lysates by a mixture of sera from AIDS patients or by the 17b antibody is shown for the wild-type and mutant envelope glycoproteins. In some lanes (+ lanes), 10 μg of sCD4 per ml was added to the lysate prior to immunoprecipitation. In each of the lanes, the more slowly migrating band represents the precursor glycoprotein, while the faster-migrating band represents the processed exterior envelope glycoprotein. (B) Precipitation of labeled envelope glycoproteins from detergent-free supernatants of transfected COS-1 cells by a mixture of sera from AIDS patients or by the 17b and 48d monoclonal antibodies. In some lanes (+ lanes), 6.6 μg of sCD4 per ml was added to the supernatant prior to immunoprecipitation.

the levels of the exterior glycoprotein in cell supernatants were greater for the mutants containing V3 deletions (Δ298-329 and Δ303-323). This result is consistent with the mild decrease in gp120-gp41 association previously observed for mutant glycoproteins with insertions or deletions in the V3 loop (42, 88).

Precipitation of the radiolabeled supernatants with the 17b and 48d monoclonal antibodies in the absence of detergent was performed (Fig. 2B). As was previously reported, deletion of the complete V1/V2 stem-loop (Δ121-203) resulted in a loss of both 17b and 48d recognition that was not altered by the addition of sCD4, which binds efficiently to the Δ121-203 mutant (58, 88). By contrast, deletion of only the V1 loop (Δ136-151) or of only the V1/V2 variable loops (Δ128-194) resulted in retention of the 17b and 48d epitopes. Figure 2B also illustrates that the complete deletion of the V3 loop (Δ298-329) significantly decreased recognition by the 17b antibody and completely disrupted recognition by the 48d antibody; however, the binding of sCD4 restores the integrity of these epitopes on the mutant glycoprotein. By contrast, a deletion of only the variable tip of the V3 loop (Δ303-323) resulted in a glycoprotein that was precipitated by both 17b and 48d antibodies in the absence of sCD4. Similarly, a mutant with deletions of both V1/V2 and V3 variable loops (Δ128-194/303-323) was recognized by both the 17b and 48d antibodies. Thus, in the context of the soluble gp120 glycoprotein, retention of the conserved elements of the V1/V2 and V3 loops preserved the integrity of the 17b and 48d epitopes.

sCD4 induction of the 17b and 48d epitopes. The addition of sCD4 to the transfected COS-1 cell lysates increased the efficiency with which the wild-type gp160 and gp120 glycoproteins were precipitated by the 17b antibody (Fig. 2A). Similar results were obtained for the Δ136-151 and Δ303-323 mutants containing deletions of the V1 and V3 loops, respectively. When the V1 and V2 loops were deleted, either alone (Δ128-194) or in combination with the V3 loop (Δ128-194/303-323), the precipitation of the envelope glycoproteins by the 17b antibody in the absence of sCD4 was significantly greater than that of the wild-type envelope glycoproteins. The addition of sCD4 to the cell lysates containing the Δ128-194 or Δ128-194/303-323 mutant did not result in further increases in the efficiency of precipitation by the 17b antibody, despite the ability of these mutant glycoproteins to bind CD4 efficiently (see below). These results suggest that the presence of the V1/V2 variable loops exerts a negative effect on the recognition of the envelope glycoproteins by the 17b antibody and that CD4 binding relieves that negative effect.

To determine more precisely the effects of the variable loop deletions on 17b and 48d recognition in the absence and presence of sCD4, we used an assay in which the gp120 glycoprotein was captured on an ELISA plate by an antibody directed against the gp120 carboxyl terminus. Supernatants derived from COS-1 transfections were used as a source of the envelope glycoproteins, and the amounts of the various envelope glycoproteins captured on the ELISA plate were normalized with an antibody (133/290) that recognizes a well-conserved linear C1 epitope (57a) present in all of the mutant glycoproteins (Fig. 3A). As shown in Fig. 3B, all of the captured envelope glycoproteins were able to bind CD4-IgG (9) with roughly comparable affinities. Thus, the abilities of the mutant glycoproteins to bind CD4 were not significantly altered by the variable loop deletions.

The binding of both the 17b and 48d antibodies to the captured wild-type gp120 glycoprotein was enhanced by the addition of sCD4 (Fig. 3C and F). The recognition of the Δ128-194 mutant glycoprotein, which contains a deletion of the V1 and V2 loops, by the 17b and 48d antibodies in the absence

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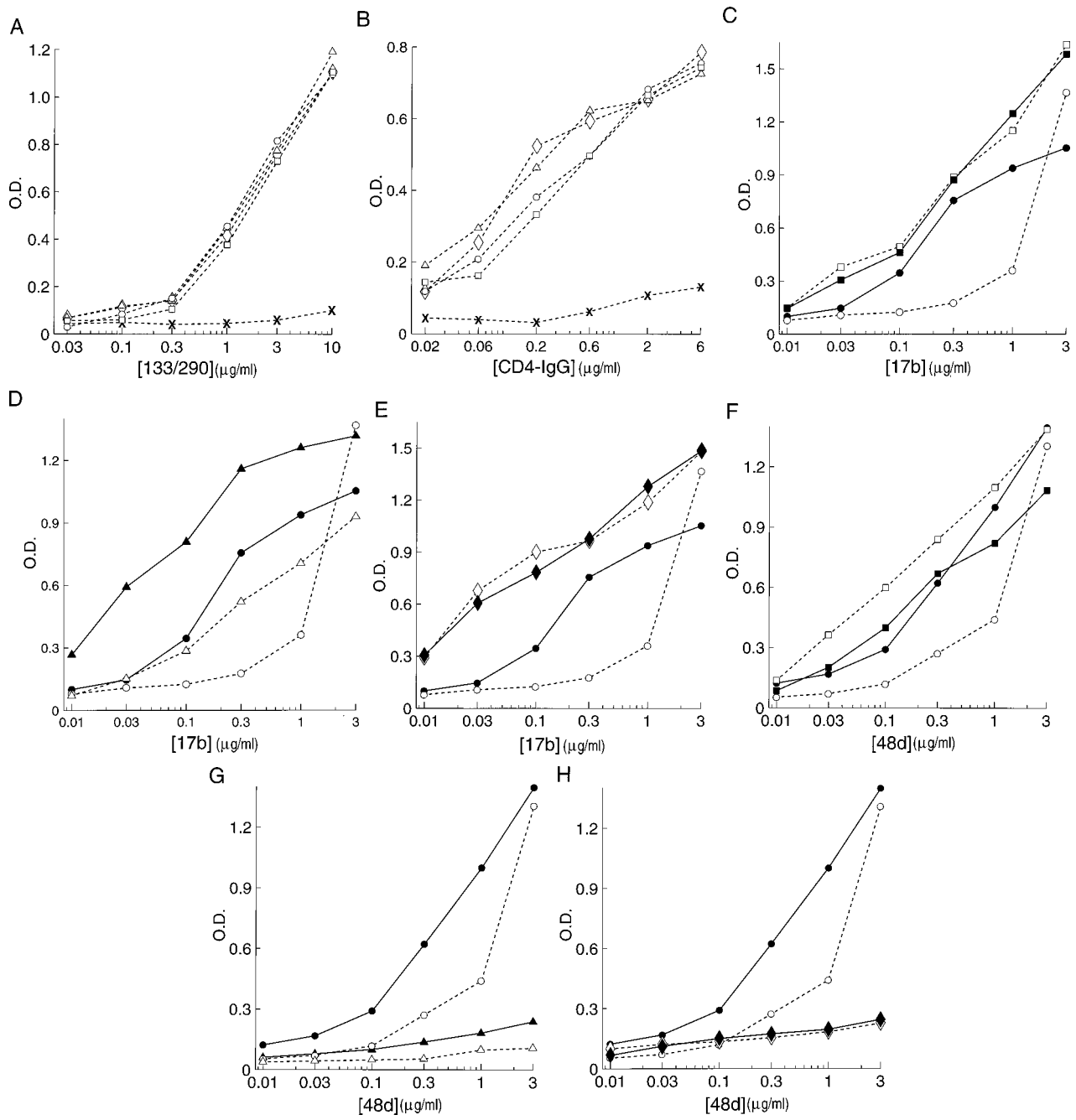

FIG. 3. Effects of variable loop deletions on antibody recognition and CD4 binding. The binding of antibodies or CD4-IgG to the wild-type or mutant envelope glycoproteins captured on an ELISA plate via a carboxyl-terminus-directed antibody is shown. Binding of the 133/290 antibody (A), CD4-IgG (B), 17b antibody (C to E), and 48d antibody (F to H) is shown for the wild-type (○, ●), Δ128-194 (□, ■), Δ303-323 (△, ▲), and Δ128-194/303-323 (◇, ◆) envelope glycoproteins. The open symbols and broken lines indicate that the experiment was performed in the absence of sCD4, while the closed symbols and solid lines indicate the presence of 5 μg of sCD4 per ml. In some experiments, the binding to wells to which no envelope glycoprotein was added (**X**) is shown. O.D., optical density.

of sCD4 was greater than or equal to that of the wild-type gp120 glycoprotein in the presence of sCD4. Addition of sCD4 did not further enhance the binding of the 17b antibody and even slightly decreased the binding of the 48d antibody to the Δ128-194 mutant (Fig. 3C and F). Deletion of only the V1 loop (Δ136-151) affected neither basal recognition by either antibody nor the enhancement of antibody binding by sCD4 relative to those of the wild-type gp120 glycoprotein (data not shown). In the ELISA format, deletion of the V3 loop (Δ303-323) disrupted 48d recognition more than 17b recognition, but addition of sCD4 increased the binding of both antibodies to the Δ303-323 glycoprotein (Fig. 3D and G). Thus, in some contexts, deletion of the variable portion of the V3 loop affects the 48d, but not the 17b, epitope. When both the V1/V2 and V3 loops were deleted (Δ128-194/303-323), the basal recognition of the mutant glycoprotein by the 17b antibody was enhanced, while that by the 48d antibody was decreased relative to those observed for the wild-type gp120 glycoprotein (Fig. 3E

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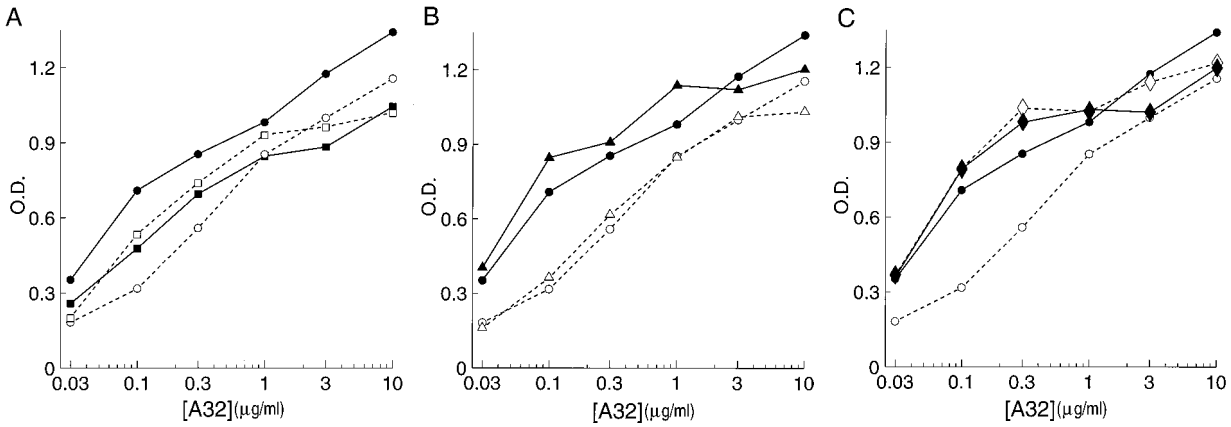

FIG. 4. Effects of variable loop deletions on sCD4-induced exposure of the A32 epitope. The binding of the A32 antibody to the wild-type (○, ●), Δ128-194 (□, ■), Δ303-323 (△, ▲), and Δ128-194/303-323 (◇, ◆) envelope glycoproteins captured on the ELISA plate is shown. The open symbols and broken lines indicate that the experiment was performed in the absence of sCD4, while the closed symbols and solid lines indicate that the experiment was performed in the presence of 5 μg of sCD4 per ml. O.D., optical density.

and H). The addition of sCD4 did not enhance the binding of either antibody to the Δ128-194/303-323 mutant. These results and the results shown in Fig. 2A suggest that the V1/V2 loop structure masks the 17b and 48d epitopes on the gp120 glycoprotein and that sCD4 enhancement of 17b and 48d antibody binding is functionally equivalent to removing this masking effect. The data also indicate that the V1 and V3 loops are dispensable for the masking of the 17b/48d epitopes and for the enhancement of 17b/48d antibody binding in the presence of sCD4.

The increased basal recognition of the Δ128-194 mutant compared with that of the wild-type gp120 glycoprotein by the 17b and 48d antibodies was also seen when the glycoproteins were captured on the ELISA plates with an antibody (133/290) directed against the first conserved (C1) region. The addition of sCD4 enhanced 17b and 48d binding to the wild-type gp120 glycoprotein captured in this manner (data not shown). These results indicate that the masking effects of the V1/V2 loops on the 17b and 48d epitopes and the induction by sCD4 binding do not depend upon the capture of the gp120 glycoprotein by an antibody directed against the carboxyl terminus.

sCD4 induction of the A32 epitope on the gp120 glycoprotein. The binding of sCD4 to the captured HIV-1 gp120 glycoprotein results in enhanced binding of the 17b and 48d antibodies, as shown above and as previously reported (79). sCD4 binding also results in smaller increases in the binding of the A32 human monoclonal antibody to the wild-type gp120 glycoprotein (Fig. 4). When both V1/V2 and V3 loops were deleted from the gp120 glycoprotein (Δ128-194/303-323), the basal level of binding of the A32 antibody was increased relative to that of the wild-type gp120 glycoprotein (Fig. 4C). For the Δ128-194/303-323 mutant, sCD4 binding did not result in an increase in the binding of the A32 antibody (Fig. 4C). These results indicate that the V1/V2 and V3 variable loops contribute to the masking of the A32 epitope on the gp120 monomer and that sCD4 binding is equivalent to demasking the A32 epitope by variable loop removal. Most of this effect is due to the V1/V2 loops, since an increase in the basal level of binding and lack of induction by sCD4 were also seen for the Δ128-194 mutant (Fig. 4A). By contrast, the V3 loop-deleted mutant (Δ303-323) exhibited basal levels of A32 antibody binding and sCD4 induction comparable to those of the wild-type gp120 glycoprotein (Fig. 4B).

Induction of the 17b and 48d epitopes by binding of the A32 antibody. The binding of the A32 antibody to the captured wild-type and mutant envelope glycoproteins resulted in an increase in the binding of the 48d and 17b antibodies (Fig. 5 and data not shown). Although deletion of the V3 loop (Δ303-329) resulted in a decrease in recognition by the 48d antibody, as previously seen, both the V1/V2 and V3 loops could be deleted without abrogating the ability of A32 to increase 48d binding (Fig. 5). Similarly, the increased binding of the 17b antibody to the gp120 glycoprotein in the presence of the A32 antibody was not dependent on the presence of the V1/V2 or V3 loops (data not shown).

Effects of variable loop deletions on sCD4-induced shedding of the gp120 glycoprotein. Previous studies indicated that the sCD4-induced shedding of the gp120 glycoprotein from the HIV-1 envelope glycoprotein complex depended upon high-affinity binding of sCD4 and also upon gp120 regions not

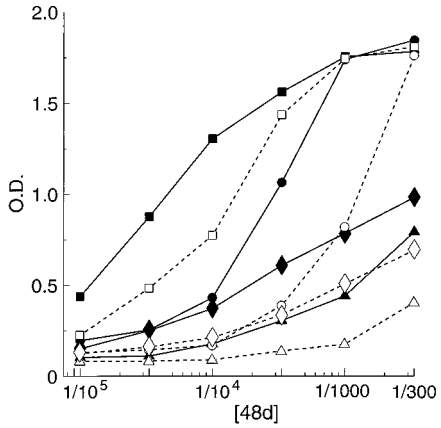

FIG. 5. Effects of variable loop deletions on A32 antibody-induced exposure of the 48d epitope. The binding of the biotinylated 48d antibody to the wild-type (○, ●), Δ128-194 (□, ■), Δ303-323 (△, ▲), and Δ128-194/303-323 (◇, ◆) envelope glycoproteins captured on an ELISA plate is shown. The open symbols and broken lines indicate that the experiment was performed in the absence of the A32 antibody, while the closed symbols and solid lines indicate that the experiment was performed in the presence of 3 μg of the A32 antibody per ml. O.D., optical density.

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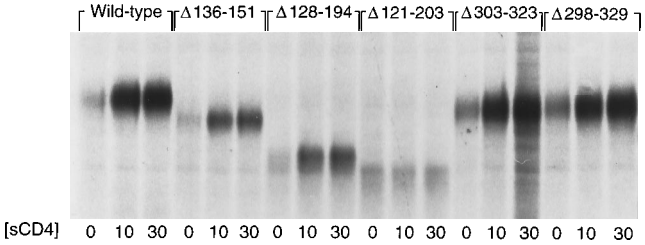

FIG. 6. Effects of variable loop deletions on shedding of the mutant envelope glycoproteins. The amounts of envelope glycoprotein precipitated from the supernatants of labeled COS-1 cells expressing the wild-type and mutant glycoproteins in the absence (0) or presence of 10 or 30 μg of sCD4 per ml are shown.

contributing directly to CD4 binding (77, 80, 88). In particular, complete deletion of the V1/V2 stem-loop structure (Δ121-203) did not decrease CD4 binding ability but completely abrogated gp120 shedding in response to sCD4 binding (88). To examine whether the V1/V2 variable loops or the conserved stem is critical for the shedding process, the ability of the Δ128-194 mutant to shed the gp120 glycoprotein as a result of sCD4 binding was examined. Figure 6 shows that the wild-type HXBc2 envelope glycoproteins demonstrated gp120 shedding in response to incubation at 37°C with sCD4. Although no gp120 shedding was observed for the Δ121-203 mutant, as previously reported (88), the Δ136-151, Δ128-194, and Δ303-323 mutant glycoproteins all exhibited gp120 shedding in response to sCD4 comparable to that seen for the wild-type envelope glycoproteins. These results indicate that the V1/V2 variable loops are not required for sCD4-induced shedding but that components in the conserved V1/V2 stem contribute to the shedding process. As was previously seen for larger V3 deletions (88), the Δ303-323 mutant shed efficiently in response to sCD4, verifying that the V3 loop is completely dispensable for sCD4-induced gp120 shedding.

Effects of variable loop deletions on envelope glycoprotein function. To determine the effects of the variable loop deletions on the function of the HIV-1 envelope glycoproteins, the wild-type and mutant glycoproteins were expressed in COS-1 cells and tested for the abilities to induce the formation of syncytia and to mediate virus entry. Compared with the wild-type glycoproteins, the mutant glycoproteins with deletions of the V1 loop (Δ136-151), the V1/V2 loop (Δ128-194), or the V3 loop (Δ303-323) exhibited relative abilities to complement virus entry into Jurkat lymphocytes of 34, 30, and 11%, respectively. Of these mutants, only the Δ136-151 and Δ128-194 envelope glycoproteins exhibited detectable levels of syncytia. Syncytium formation is typically more sensitive to amino acid changes in the HIV-1 envelope glycoproteins than is virus entry (8, 31, 80). Additional mutant glycoproteins containing more-extensive deletions involving the entire V3 (Δ298-329) loop or combinations of V1/V2 and V3 deletions were included in these assays for comparative purposes. These mutants did not exhibit detectable ability to mediate syncytium formation, consistent with the lower abilities of these mutants to mediate virus entry into Jurkat lymphocytes (Table 1) (88).

TABLE 1. Functional properties of the HIV-1 envelope glycoprotein mutants

Envelope glycoprotein	Syncytium-forming ability[a] (%)	Replication complementation[b] (%)
Wild-type	100	100
Δ136-151	14	34
Δ128-194	4	30
Δ303-323	<1	11
Δ298-329	<1	6
Δ128-194/303-323	<1	6
Δ121-203/298-329	<1	4

[a] Syncytium-forming ability with SupT1 target cells was measured as described in Materials and Methods and is normalized to that of the wild-type envelope glycoproteins (100%). The syncytia induced by the Δ136-151 and Δ128-194 mutants were smaller than those formed by the wild-type envelope glycoproteins.

[b] Complementation of virus entry into Jurkat lymphocytes was performed as described previously (31, 80). The CAT activity observed with the pSVIIIenvΔKS control plasmid was subtracted from the CAT activity seen for each of the plasmids expressing the wild-type mutant envelope glycoproteins, and the resultant value was normalized to that observed for the wild-type envelope glycoproteins (100%). The values shown are from a typical experiment. The relative complementation ability obtained from three independent experiments differed from the values shown by less than 5%.

DISCUSSION

The observed enhancement of HIV-2 and SIVagm envelope glycoprotein-mediated fusion events by sCD4 suggested that receptor binding might trigger positive events in virus entry not related to virus attachment to the target cell (2, 14). More recently, sCD4 enhancement of infection by certain strains of HIV-1 has been observed (74), indicating that receptor-mediated activation may apply to the primate immunodeficiency viruses in general. Several effects of CD4 binding on the conformation of the HIV-1 envelope glycoproteins have been described, although not all of these changes appear to represent necessary events in virus entry (25, 30, 39, 50, 65, 66, 69, 79). Understanding the molecular basis of CD4-induced changes in the viral envelope glycoproteins will be critical in distinguishing between functionally relevant and irrelevant events. The increased exposure of the 17b and 48d epitopes represents an attractive candidate for a functionally relevant CD4-induced conformational change, since these epitopes are neutralization targets (79) and may be located in gp120 regions important for virus entry.

The discontinuous regions recognized by the 17b and 48d antibodies are among the most conformation-sensitive epitopes on the native HIV-1 gp120 glycoprotein. These epitopes, for example, are completely disrupted in some buffers containing ionic detergents that do not affect the integrity of other discontinuous epitopes, such as those overlapping the CD4 binding site (79). Perhaps as a result of this sensitivity to subtle changes in gp120 conformation, some of the effects of deletions in the variable loops on the 17b and 48d epitopes are dependent upon the context in which binding of the antibodies to the gp120 glycoprotein is measured. The binding of other ligands, for example, may restrict the number of conformations available to gp120 mutants, which in turn can affect the degree to which certain changes disrupt the 17b and 48d epitopes. sCD4 binding has been shown to restore 17b or 48d recognition of several mutant gp120 glycoproteins that, as free molecules in solution, were unable to be precipitated by these antibodies (Fig. 2) (81). In this study, the variable-loop-deleted gp120 glycoproteins were examined in two contexts, as free molecules in solution and as glycoproteins captured on ELISA plates by an antibody directed against the gp120 carboxyl terminus. Perhaps related to the effects of binding the capture antibody to the gp120 glycoprotein, some differences in the effects of variable loop deletions on the 17b and 48d epitopes were seen in these two assay systems. First, the studies performed using envelope glycoproteins in solution confirm that the highly conserved stem in the V1/V2 stem-loop structure can influence the integrity of the 17b and 48d epitopes. Re-

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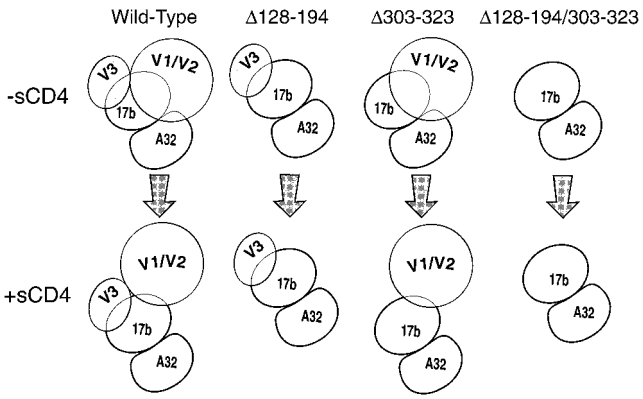

FIG. 7. Model for variable-loop-mediated masking of the 17b and A32 epitopes and for the effects of sCD4 binding. −sCD4, in the absence of sCD4; +sCD4, sCD4 binding.

tention of this stem preserves recognition of a V1/V2 variable-loop-deleted glycoprotein by both antibodies, whereas deletion of the complete V1/V2 stem-loop structure (Δ121-203 in Fig. 2B) resulted in a complete loss of precipitation by both 17b and 48d antibodies. By contrast, when the gp120 glycoprotein is captured on an ELISA plate with an antibody directed against the C terminus, the complete deletion of the V1/V2 structure (Δ119-205 or Δ121-203) disrupted recognition by the 48d antibody but not by the 17b antibody (data not shown). This result indicates that these are subtle differences in the 17b and 48d epitopes; the V1/V2 stem is not required for the binding of the 17b antibody in all contexts, whereas the V1/V2 stem appears to be more critical for 48d binding. A second example in which the context influenced the results was found in the effects of V3 loop deletions on recognition of the gp120 glycoprotein by the 17b and 48d antibodies. Deletion of the entire V3 loop (Δ298-329) resulted in a significant loss of precipitation of the deleted glycoprotein from detergent-free solutions by both 17b and 48d antibodies. The binding of sCD4 to this V3-deleted mutant fully restored the ability to be precipitated by both 17b and 48d antibodies (Fig. 2B). The more conservative deletion of the V3 loop (Δ303-323) did not affect the ability of either the 17b or 48d antibody to precipitate the mutant glycoprotein in solution (Fig. 2B). The same conservative deletion, however, considerably decreased recognition by the 48d, but not the 17b, antibody, when the mutant glycoprotein was captured on the ELISA plate with the C-terminus-directed antibody (Fig. 3D and G). These results suggest that, in some contexts, the 48d epitope is more sensitive to changes in the V3 loop than is the 17b epitope. This observation is consistent with the greater effect of V3-directed antibodies on the binding of the 48d antibody than on the binding of the 17b antibody (63a). Apparently, critical components of the 48d epitope can be altered by changes in the V3 loop.

The more conservative nature of the deletions in the major gp120 variable loops made in this study compared with those in a previous study (88) allowed retention of the integrity of the 17b and 48d epitopes in most contexts. This, in turn, allowed us to investigate the contribution of the variable loops to changes in the exposure of the 17b, 48d, and A32 epitopes induced by the binding of sCD4. A model derived from these studies is presented in Fig. 7. Removal of the V1/V2 loops (Δ128-194) resulted in an increased exposure of the 17b and 48d epitopes to an extent even greater than that seen upon sCD4 binding to the wild-type gp120 glycoprotein. The binding of sCD4 resulted in no further increase in exposure of the 17b and 48d epitopes on the V1/V2-deleted mutant glycoprotein. Therefore, the V1/V2 variable loops appear to mask the 17b/48d epitopes on the monomeric gp120 glycoprotein and the major effect of sCD4 binding is to demask these epitopes by inducing conformational changes in the V1/V2 structure. Selective removal of the V1 loop alone (Δ136-151) did not increase the exposure of the 17b and 48d epitopes, and the binding of sCD4 to this mutant glycoprotein resulted in an increased exposure of both epitopes. This result suggests that the V2 loop, but not the V1/V2 stem, is sufficient to mask the 17b and 48d epitopes and may be more important than the V1 loop in mediating sCD4-induced changes. Previous studies have suggested that sCD4-induced movement of the HIV-1 gp120 V2 loop could result in selective masking of some conformation-dependent V2 epitopes (49, 54, 69). Furthermore, the V2 tip has been implicated in fusion-related virus entry functions modulated by CD4 binding (13, 75). The potential relevance of the exposure of the 17b and 48d epitopes as markers for functionally important CD4-induced alterations in the HIV-1 envelope glycoproteins is supported by the association of the V2 loop with both processes.

The induction by sCD4 of the exposure of the A32 epitope was also dependent upon the gp120 major variable loops (Fig. 7). When the V1/V2 and V3 loops were removed (Δ128-194/303-323), a demasking of the A32 epitope on the monomeric gp120 glycoprotein was observed. As was seen for the 17b and 48d epitopes, sCD4 binding did not result in further exposure of the A32 epitope. While the smaller magnitude of sCD4-induced exposure of the A32 epitope compared with those of the 17b and 48d epitopes makes a precise comparison among the mutants more difficult, it appears that the V1/V2 structure rather than the V3 loop plays the major role in this induction as well. The A32 epitope is a poorer neutralization target than are the 17b and 48d epitopes, probably because the former epitope is not as exposed on the oligomeric envelope glycoproteins (87). The exposure of the A32 epitope on the oligomeric envelope glycoproteins in the presence of CD4 and the functional relevance of such exposure will be the subject of future studies.

On the monomeric HIV-1 gp120 glycoprotein, the binding of the A32 antibody increases the subsequent binding of the 17b and 48d antibodies. Our results indicate that this induction occurs via a mechanism that is not dependent upon the presence of the V1/V2 or V3 loops. Therefore, the increases in exposure of the 17b and 48d epitopes induced by sCD4 and by the A32 antibody proceed via different mechanisms.

Examination of the functional abilities of the mutant glycoproteins revealed that the large variable segments of the V1/V2 and V3 loops are not absolutely required for HIV-1 entry, although they contribute to the efficiency of this process. Syncytium-forming ability was more dramatically affected than was virus entry, as has been previously seen for other HIV-1 envelope glycoprotein mutants with defects in membrane fusion (8, 31, 75, 80). The decreased functional activities of the variable-loop-deleted glycoproteins are probably not due solely to effects on exposure of the 17b and 48d epitopes, since the V1-deleted mutant is not apparently altered in this parameter yet exhibits partially attenuated virus entry and cell-cell fusion. The retention of some function by the mutants with more-conservative variable loop deletions contrasts with the loss of function observed for mutants with more-extensive V1/V2 or V3 truncations (Table 1) (88). This result suggests that some of the loss of function associated with the latter mutants may arise from secondary effects of the deletions on conserved structures near or within the 17b and 48d epitopes. While the existence of neutralization escape mutants (79) indicates that the preser-

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vation of the 17b and 48d epitopes per se is not essential for HIV-1 entry, conserved structures overlapping these epitopes may represent critical components in post-receptor binding events.

Previous studies indicated that complete deletion of the V1/V2 stem-loop structure (Δ121-203) resulted in a mutant glycoprotein that bound CD4 at a level comparable to that of the wild-type envelope glycoprotein but did not exhibit sCD4-induced shedding of the exterior envelope glycoprotein (88). Here we show that retention of the V1/V2 stem in the Δ128-194 mutant allows sCD4-induced gp120 shedding to occur efficiently. Thus, even with the V1/V2 loops deleted, CD4 binding mediates changes in the conformation of the HIV-1 envelope glycoproteins through a mechanism dependent upon the conserved V1/V2 stem. The relevance of such changes to HIV-1 entry remains uncertain. The existence (4, 77) of fusion-defective gp120 mutants that shed the exterior glycoprotein efficiently upon CD4 binding, such as V3 loop-deleted mutants, makes it highly unlikely that dissociation from the gp120 glycoprotein represents the sole postattachment function of the gp120 glycoprotein during the virus entry process. The existence of fusion-competent mutant glycoproteins that do not shed the gp120 glycoprotein in response to sCD4 provides a strong argument against the necessity of shedding in HIV-1 entry (77). Nonetheless, it remains possible that some overlap exists between conformational changes in the gp120 glycoprotein related to shedding and those related to virus entry. Future structure-function analysis of the HIV-1 envelope glycoproteins should help to define these relationships and to increase our understanding of the mechanism of virus-cell fusion.

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