CD4-binding regions of human immunodeficiency virus envelope glycoprotein gp120 defined by proteolytic digestion

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ABSTRACT The gp120 envelope glycoprotein of human immunodeficiency virus type 1 binds the cell surface protein CD4 with high affinity. Here we report the use of proteolysis to define regions of gp120 involved in CD4 binding. Cleavage of gp120 with Staphylococcus aureus V8 protease at residue 269 or with trypsin at residue 432 destroys CD4 binding. These same sites are protected from proteolytic cleavage by bound CD4. Cleavages at 64, 144, 166, 172, and 315 do not affect binding and are not protected by bound CD4, indicating that these regions are not critical for binding CD4. All proteolytic fragments found in coprecipitates with CD4 were covalently associated via disulfides and comprised complete gp120 molecules. Previous conclusions by Nygren et al. [Nygren, A., Bergman, T., Matthews, T., Jornvall, H. & Wizsell, H. (1988) Proc. Natl. Acad. Sci. USA 85, 6543-6546] that both large and small (95-kDa and 25-kDa) V8 proteolytic fragments bind CD4, independently, are not distinguished by their experiments from the result found here that small fragment immunoprecipitates with CD4 while disulfide-linked to the larger fragment.

Human immunodeficiency virus type-1 infection of CD4-positive lymphocytes is initiated by the binding of the viral envelope glycoprotein to the cell surface protein CD4 (1–3). The env gene product gp160 is posttranslationally cleaved to give gp120 and gp41 as components of the mature protein (4). CD4 binding is mediated by gp120 and directs the virus’ CD4-positive cell tropism, a major indicator of acquired immune deficiency syndrome, AIDS.

Extensive directed mutagenesis indicates that most mutations which perturb CD4 binding occur in the C-terminal half of gp120 (5–9, 11). However, some reports suggest that N-terminal sequences may also be important for binding (11, 12). The existence of a discrete C-terminal CD4-binding domain of gp120 was suggested by Nygren et al. (13), who concluded that both 95- and 25-kDa C-terminal proteolytic fragments of gp120 independently retained the ability to bind CD4.

Here we report the use of proteolysis to define regions of gp120 involved in binding CD4. Proteolytic cleavages at residues 269 and 432 and within the N-terminal region, possibly at residue 91, are identified as important in influencing CD4 binding. Regions around residues 64, 144, 166, 172, and 315 appear not to be critical for CD4 binding. Proteolytic fragments found in coprecipitates with CD4 were covalently associated via disulfides and comprised complete gp120 molecules. Evidence is presented that previous results suggesting independent CD4 binding of both large and small (95-kDa and 25-kDa) fragments produced by cleavage with the Staphylococcus aureus V8 protease can be explained by the covalent association of the two fragments via disulfide bonds in the CD4 precipitates.

MATERIALS AND METHODS

Proteins. Purified recombinant gp120 and recombinant soluble CD4 (14) were kindly supplied by Biogen. The gp120 was produced in Spodoptera frugiperda insect cells using a recombinant baculovirus (Autographa californica). The construction and expression of the recombinant virus have been described (15). The recombinant baculovirus expresses a gp120 molecule of ≈100 kDa that is derived from the envelope of the HXB2 strain of human immunodeficiency virus type 1 and includes amino acids 44–500 (HXBCG2, GenBank; ref. 16). The N terminus of this protein consists of amino acids 35 to +1 of tissue plasminogen activator (tPA) (17), which includes the signal sequence. The mature N terminus of purified gp120 indicates there to be a mixture of either 5 or 15 (70-30 ratio) tPA amino acids (17) prior to gp120-specific amino acids, which start with residue 44 [gp120 (HXBCG2) numbering includes the signal peptide].

Digestions with Trypsin and S. aureus V8 Protease. For a typical trypsin digestion, 2 μg of native, unreduced gp120 was incubated with 1% (wt/wt) trypsin (gp120) trypsin (Boehringer Mannheim) at 25°C for 1 hr in 20 μl of phosphate-buffered saline (PBS): 138 mM NaCl and 2.7 mM KCl in phosphate buffer, pH 7.4) containing 20 mM CaCl2. For V8 protease (Boehringer Mannheim) digestion, 2 μg of native, unreduced gp120 was incubated with 12.5% (wt/wt) V8/gp120V8 protease at 37°C for 2 hr in 20 μl of 0.1 M ammonium bicarbonate (Sigma). For CD4 precipitation experiments, the digestions were stopped by the addition of a molar excess of α2-macroglobulin (Boehringer Mannheim). Concentrated (10×) SDS gel loading buffer (18) was added to digest aliquots to make 1× buffer. Samples were resolved in an SDS/12.5% polyacrylamide gel with a discontinuous buffer system (18).

CD4 Precipitations. Samples of digests (usually half of the digest), after the addition of α2-macroglobulin, were made up to 1 ml with PBS supplemented with 0.1% bovine serum albumin (BSA) and incubated overnight with soluble CD4 crosslinked to Sepharose beads [20 μl of a 50% suspension per microgram of gp120; made using methods described by the manufacturer (Sigma)] at 4°C on a rocking platform. After three washes with 1 ml of PBS, bound material was eluted from the beads with 2× gel loading buffer and resolved in an SDS/12.5% polyacrylamide gel.

CD4 Protection Experiments. Digestions were performed as usual but with the addition of a 10-fold molar excess of soluble CD4 (10 μg of CD4 per 2 μg of gp120; the molecular mass of the soluble CD4 is about 50 kDa). In control digestions an equivalent mass of bovine serum albumin was added in place of the CD4. The amount of protease relative to the total amount of protein (i.e., gp120 plus CD4 or BSA) was 1% (wt/wt) for trypsin and 12.5% (wt/wt) for V8 protease. The digestions were stopped by the addition of gel loading buffer (18) and resolved in an SDS/12.5% polyacrylamide gel.

Abbreviation: BSA, bovine serum albumin.
**Western Blots.** Digested material resolved in an SDS/polyacrylamide gel was transferred electrophoretically onto nitrocellulose (18). The blot was probed using a rabbit polyclonal antibody against the recombinant gp120 (kindly supplied by Biogen). Bound antibody was detected using a goat anti-rabbit IgG antibody/alkaline phosphatase conjugate and visualized using the recommended substrates (Immunoall, GIBCO/BRL).

**N-Terminal Amino Acid Analysis.** The trypsin and V8 digestions were scaled up to 100 μg of gp120. The digestion conditions were as described above. Digested material was resolved in a reducing SDS/12.5% polyacrylamide gel and transferred electrophoretically onto an Immobilon membrane (Millipore). Proteins were visualized with Coomassie blue stain, isolated using a scalpel blade, and subjected to automatic Edman degradation in an Applied Biosystems 470A gas-phase sequencer equipped with a 900A data system. The resulting phenylthiohydantoin amino acid derivatives were analyzed using an Applied Biosystems 120A amino acid analyzer equipped with a PTH C18 column (2.1 × 22 mm).

**RESULTS**

Comparison of reducing and nonreducing SDS/polyacrylamide gel profiles of gp120 digested (unreduced) with either V8 protease or trypsin indicates that the native protein is cleaved at several sites by these enzymes but that many of the resultant fragments remain associated via one or more of the nine disulfide bonds in gp120 (19). After V8 digestion of gp120, eight isolated bands are resolved in a reducing SDS gel, compared with only three or four major bands under nonreducing conditions (Fig. 1, lanes 1 and 4). After trypsin digestion, despite the appearance of six major cleavage products on a reducing gel, the digested protein remains intact when not reduced, migrating as a single band that corresponds to full-length gp120 (Fig. 1, lanes 6 and 8).

![Fig. 1](image-url) CD4 precipitations of digested gp120. Shown are Western blots of proteins resolved on reducing and nonreducing SDS/polyacrylamide gels (as indicated). (Left) Results of V8 digestion of gp120 and subsequent CD4 precipitation of digested material. Lane 1, the eight proteolytic fragments resolved under reducing conditions after V8 digestion (fragments indicated with arrows 1–8); lane 2, V8-digested protein that precipitated with CD4 (analyzed after reduction); lane 3, undigested gp120; lane 4, V8-digested gp120, analyzed unreduced; lane 5, V8-digested gp120, CD4-precipitated and analyzed unreduced. Only V8 fragments 1, 5, 6, and 7 coprecipitate with CD4 (lane 2). These fragments are linked by disulfides and constitute an intact molecule—i.e., a single band of the same molecular mass as intact gp120 (compare undigested gp120, lane 3, with CD4-precipitated protein, lane 5). (Right) Results of trypsin digestion of gp120 and subsequent CD4 precipitation of digested material. Lane 6, the six trypsin fragments resolved under reducing conditions after digestion (indicated with arrows 1–6); lane 7, trypsin-digested protein precipitated by CD4, analyzed reduced (note that trypsin fragments 1, 2, 4, and 5 coprecipitate with CD4 whereas fragment 6 does not); lane 8, nonreducing gel indicating that all the trypsin fragments are linked by disulfides; lane 9, CD4 precipitation of trypsin-digested gp120 analyzed unreduced, showing that only full-length gp120 binds CD4; lane 10, a longer digestion of gp120 with trypsin; lane 11, CD4 precipitation of sample from a longer digestion, illustrating that fragment 3 is not found in coprecipitates with CD4. The precipitated bands are more faint in lanes 10 and 11, as prolonged digestion destroys CD4 binding of most of the gp120 present.
On a reducing SDS gel all the clearly visible trypsin fragments (fragments 1, 2, 4, and 5), except fragment 6 (produced by cleavage at 432), are found in precipitates with CD4 (Fig. 1, lane 7). If the trypsin digestion is allowed to proceed further, whereupon fragment 3 can be seen more clearly (lane 10), it is still not found in precipitates with CD4 (lane 11). (After prolonged digestion, the precipitable bands become fainter as the amount of digested material still able to bind is greatly reduced. Fragment 3 appears faint on a Western blot, probably due to its poor antigenicity, as a Coomassie stain of similar gels indicated it to be present in similar amounts to the other fragments.) These results indicate that cleavages at 146, 166, and 315, which produce trypsin fragments found in coprecipitates with CD4 (fragments 1, 2, 4, and 5), do not affect CD4 binding (Fig. 2). However, it appears that cleavages around 432, which result in fragments 3 and 6, fragments that are not found in CD4 precipitates, destroy CD4 binding. The size of fragment 6 suggests that it extends to the C terminus of gp120 and that it is the cleavage at 432 (its N terminus) that destroys CD4 binding (Fig. 2). The N-terminal cleavage site of fragment 3 (residue 315, Fig. 2) cannot affect CD4 affinity, because that site is shared by fragment 2c, which is found in CD4 precipitates. This suggests that the C terminus of fragment 3, which the fragment’s size indicates is near residue 432, must be the site affecting CD4 affinity (Fig. 2).

**CD4 Protection: Cleavage in the Presence of CD4.** After trypsin digestion in the presence of CD4, all the trypsin fragments were produced except fragment 6 (Fig. 3A). Similarly, V8 fragments 2, 3, and 4 were not produced after digestion in the presence of CD4 (Fig. 3B). These same trypsin and V8 fragments were not found in gp120 molecules that retained CD4 affinity in the previous experiments. These observations provide further evidence that the cleavages at 269 and 432 directly affect CD4 binding (see above). The cleavage site that produces the N terminus of trypsin fragment 6 have not sequenced the C termini of the proteolytic fragments in Fig. 2, we cannot identify the C-terminal cleavages of fragments 5, 6, and 7, which do not affect CD4 binding, but from the size of those fragments the cleavage sites must be in the region of residue 172. V8 fragments 2, 3, 4, and 8 are not found in cleaved gp120 molecules precipitated by CD4 (Fig. 1, compare lane 2 with lane 1), indicating that some of the cleavages producing these fragments destroy CD4 affinity. The size of fragment 2, which is not found in CD4 precipitates, indicates that it probably extends to the C terminus of gp120, which argues that the cleavage at 269 (its N terminus) destroys CD4 binding (Fig. 2, also see below). Furthermore, the cleavages at the C terminus of fragments 3 and 4 must destroy binding, because they have the same N terminus as V8 fragment 1, which is found in coprecipitates with CD4 (Fig. 1, lane 2). The sizes of fragments 3 and 4 indicate that their C termini may be near residue 269 (Fig. 2). Although faint in the nonprecipitated sample, V8 fragment 8 was reproducibly absent from precipitates with CD4 (Fig. 1, lane 2), which indicates that cleavages associated with this fragment (residue 91 at the N terminus) may affect CD4 binding.

**Fig. 3.** Protection of cleavage sites by bound CD4. Western blots of proteins resolved in reducing SDS/12.5% polyacrylamide gel after digestion with either V8 or trypsin in the presence of CD4 (10-fold molar excess) or BSA (equivalent mass to CD4). (A) Lane 1, digestion of gp120 with trypsin in the presence of CD4; lane 2, digestion of gp120 in the presence of BSA. In the presence of bound CD4 all of the fragments are produced except fragment 6 (indicated with an asterisk). (B) Lane 1, digestion of gp120 with V8 in the presence of BSA; lane 2, digestion of gp120 with V8 in the presence of CD4. In the presence of bound CD4, fragments 1, 5, 6, and 7 are produced, but fragments 2, 3, and 4 are not (indicated with asterisks). The fragments not produced in the presence of CD4 are the same fragments that do not precipitate with CD4 (see Fig. 1, lane 7 and lane 2), indicating that the cleavage sites associated with these fragments, residues 269 and 432, are involved in CD4 binding (see Fig. 2 for summary).
ment 6, at residue 432, appears to be protected by CD4, resulting in a band from the sum of fragments 3 and 6 at or below band 2 (Fig. 3A, lane 1). The protection of the C-terminal cleavage site (Fig. 2), which must be near the C terminus of gp120, could only have caused the production of a slightly larger fragment near 4 and 5 that is not observed (Fig. 3A, lane 1).

The size of V8 fragment 2 indicates that it extends to or nearly to the C terminus of gp120 (Fig. 2). The disappearance of V8 fragment 2 into a fragment the size of 1 (Fig. 3B, lane 2) argues that the N-terminal cleavage site at 269 is protected (Fig. 2). The N termini of V8 fragments 3 and 4 are the same as that of fragment 1 (produced by cleavage at 172; Fig. 2). Fragment 1 is still produced in the presence of CD4. Thus the cleavage sites at the C termini of fragments 3 and 4 must be protected by CD4. They are proximal to 269, the site implicated by fragment 2, above. Supporting this conclusion, the presence of CD4 results in an increased yield of fragment 1 (Fig. 3B, lane 2) as expected because blocking cleavage at 269 would be expected to prevent formation of fragments 2, 3, and 4 and to result in more fragment 1 (Fig. 2; Fig. 3B, lane 2).

V8 fragment 8 was not found in coprecipitates with CD4 (see above). However, CD4 protection of the cleavage sites associated with this fragment was ambiguous due to fragment 8’s faint appearance on Western blots, which resulted in it not always being observed from experiment to experiment. Even when the digestions were allowed to proceed longer (as for trypsin fragment 3, above), it was not possible to demonstrate protection at the fragment 8 sites (i.e., V8 protease sites around position 91; see Discussion). Silver staining of similar gels did not resolve this problem, as cleavage products of CD4 and of the V8 and trypsin proteases themselves obscured the gp120 fragments. All CD4 protection digestions included a 10-fold molar excess of CD4 in case CD4 was itself affected by the protease. Equivalent quantities of BSA were used in place of soluble CD4 in control experiments (Fig. 3, lanes 2).

**DISCUSSION**

Digestion of gp120 with trypsin and V8 protease suggests that gp120 molecules cleaved at residues 432 (trypsin) and 269 (V8) are unable to bind CD4. In addition, digestion of gp120 with these enzymes in the presence of CD4 demonstrates that these same sites are protected from digestion. The CD4 protection experiments argue against the interpretation that the proteolytic cleavages at 432 and 269 act indirectly to affect CD4 binding, and favor the interpretation that these residues are in or near the “footprint” of CD4 on gp120.

Most reported mutations in gp120 indicate that at least the C-terminal region extending from residues 381 to the C terminus (S11) is critical for CD4 binding (summarized in Fig. 4; refs. 5–9, 11). The cleavage at 432 is therefore within a region that has been shown to be important in binding CD4. Additionally, residues 397–439 (corresponding to 413–456 here), which include the trypsin site at 432, correspond to the binding site for a gp120-specific monoclonal antibody that blocks CD4 binding (5). The cleavage at 269 by V8 protease does not occur within the C-terminal CD4-binding region just described. However, directed mutagenesis of conserved amino acids of gp120 showed that changing residue 257 from threonine to arginine significantly reduced CD4 binding (11). The cleavage at 269 is proximal to 257 and therefore provides additional support for the importance of this region in CD4 binding.

The N-terminal V8 fragment 8 (N terminus at residue 91) was not found in coprecipitates with CD4, suggesting that residues in the N terminus may also be involved in CD4 binding. (For technical reasons we were unable to determine whether or not CD4 protected the sites associated with this fragment.) Some small effects on CD4 binding caused by single amino acid changes at residues 80 and 120/121 (11) and deletion of residues in this region, which destroys CD4 binding (9, 11), are consistent with our results but do not distinguish a direct effect on binding from an indirect effect on structure.

Cleavages by trypsin and V8 protease at residues 64, 146, 166, 172, and 315 did not affect CD4 binding and were not prevented by bound CD4. This suggests that regions around these sites (in the N terminus and the V1, V2, and V3 regions) are not critical for CD4 binding. Most of these sites occur in regions of variable amino acid sequence or in regions that are known to be immunogenic (Fig. 4). In another study, we have observed that deletion of N-terminal regions as well as sequences that include the V1, V2, and V3 variable sequence regions of gp120 does not affect CD4 affinity (S.R.P., M. D. Rosa, J.J.R., and D.C.W., unpublished work).

After V8 digestion, we have observed that both a large C-terminal fragment (fragment 1) and three smaller N-terminal fragments (fragments 5, 6, and 7) are found in coprecipitates with CD4 but that these fragments constitute full-length molecules, remaining covalently associated via disulfide bonds (Fig. 1, lanes 2 and 5). Similarly, Nygren et al. (13) observed that after V8 digestion, both large, 95-kDa and small, 25-kDa fragments were found in molecules with CD4 affinity. They suggested, speculatively, that the 25-kDa fragment originated from the C terminus of the molecule. The disulfide structure of gp120 (19) indicates that the 95-kDa fragment reported by Nygren et al., produced by V8 cleavage at residue 142 (in ref. 13; residue 172 here), would be linked by disulfide bonds to a 25-kDa N-terminal fragment. This would result in both the large 95-kDa and an N-terminal 25-kDa fragment being present in single molecules that coprecipitate with CD4. Nygren et al. (13) did not determine whether or not their two fragments were linked by disulfide bonds when active. Therefore their conclusion that
a discrete 25-kDa fragment retained CD4 binding affinity was not distinguished in their experiments from the result found here, that the small fragment(s) are only, so far, indirectly associated with CD4 binding activity when disulfide-linked to the remainder of gp120. [Despite differences between the gp120 preparations that were used, 7 amino acid substitutions between BH10 and HXB2 and potential glycosylation differences, the cleavage map determined here is clearly similar to the partial map determined by Nygren et al. (13). The large CD4-precipitable fragment observed here has the same N terminus as the 95-kDa fragment previously reported. Smaller fragments, not found in molecules with CD4 affinity, contained the same N terminus as the large fragment, again as reported. The lack of tyrosine residues in close proximity to residue 270, which corresponds to the N terminus of the second-largest fragment observed here, is consistent with Nygren et al. being unable to identify their second-largest fragment by sequencing iodinated tyrosines. Smaller cleavage products, observed here and previously, correspond to N-terminal peptides that include the mature N terminus.]

Proteolytic fragments not directly involved in CD4 binding could be passively coprecipitated with CD4 as a result of covalent association with a CD4-binding fragment. Attempts were made to wash digested gp120 after CD4 precipitation with reducing solutions containing dithiothreitol. However, we were unable to dissociate any fragments by this method. In summary, regions in the C-terminal half of the gp120 molecule near the cleavage sites at 269 and 432 are involved in CD4 binding. These conclusions derive from two complementary experiments, the loss of CD4 affinity resulting from proteolytic cleavages and the protection of the cleavage sites in the presence of excess CD4, which together argue in favor of the interpretation that 269 and 432 are in the CD4 footprint on gp120, rather than more complicated indirect interpretations or actions at a distance due to CD4 binding, which might explain either result separately. There appears to be some indication that N-terminal regions may also be involved in binding, both from genetic mutations previously reported (see Fig. 4; refs. 9, 11, 12) and from our observation that V8 cleavages between residues 91 and 172 may affect binding. Previous reports have demonstrated that the expression of mutant gp120 molecules missing the first 164 amino acids from the mature N terminus results in a protein unable to bind CD4 (10). Similarly, we have observed that the expression of a protein corresponding to the large V8 fragment results in a protein that binds CD4 very weakly. However, coexpression of this protein fragment with an N-terminal fragment results in both fragments being coprecipitated with CD4 (S.R.P., M. D. Rosa, J. J.R., and D.C.W., unpublished work). This “complementation” indicates that the N terminus may be involved directly in binding CD4 or that it is at least required for the correct folding of gp120.

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