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The N-terminal region of the human immunodeficiency virus envelope glycoprotein gp120 contains potential binding sites for CD4

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ABSTRACT **Human immunodeficiency virus (HIV) vaccines targeted at blocking HIV–CD4 interactions are expected to be less affected by the sequence heterogeneity of HIV than those targeted at variable regions of the envelope outercoat glycoprotein, gp120. All potential CD4 binding sites identified thus far in HIV are localized in the C-terminal region of gp120. In this study we demonstrate that the N-terminal region of gp120 also contains conserved residues critical for binding to CD4 and that gp120–CD4 interactions can be blocked by an antiserum with binding specificity to an N-terminal region of gp120. These results suggest that not all potential CD4 binding sites are present in the C-terminal region of gp120 and that an alternative HIV vaccine development strategy may have to include the N-terminal gp120 region as a component to raise effective CD4-blocking antibodies.**

The binding of the envelope glycoprotein gp120 to CD4 is a critical step leading to human immunodeficiency virus (HIV) infection (1–3). A major goal of HIV vaccine development, therefore, is to block the gp120–CD4 interaction, which has a binding constant of greater than 10^9 M (4, 5). HIV-1 gp120 contains conserved coding sequences separated by sequences that differ among different viral isolates (Fig. 1*A*). Several previous studies have localized potential CD4 binding sites on HIV to the C-terminal conserved region of gp120 (4, 11–13). (*i*) Lasky *et al.* (4) mapped the epitope recognized by monoclonal antibodies that blocked CD4–gp120 interactions *in vitro* to a conserved sequence in the C-terminal region of gp120. The amino acid residues that were critical for CD4 binding were subsequently identified using site-directed mutagenesis (4, 12). (*ii*) By using the approach of linker-scanning, Kowalski *et al.* (11) identified three noncontiguous sequences in the C-terminal region of gp120 as critical for CD4 binding. (*iii*) Linsley *et al.* (13) found that the deletion of 44 amino acid residues from the C terminus of gp120 caused reduced binding to CD4, indicating that at least some of these amino acid residues are involved in CD4 binding. (*iv*) Nygren *et al.* (14) suggested that a C-terminal 25-kDa gp120 peptide was sufficient to bind to CD4. The mutational analyses of the first three studies are sufficient to indicate involvement of the C-terminal region of gp120 in CD4 binding; however, they did not rule out the possibility that other regions of gp120 may also be involved in CD4 binding. Likewise, although the study by Nygren *et al.* (14) concludes that a 25-kDa C-terminal gp120 peptide is involved in CD4 binding, it too leaves open the possibility that another 25-kDa gp120 fragment mapped to the N-terminal region of gp120 participates directly in CD4 binding.

The present study was designed to explore the possibility that the N-terminal region of gp120 may contain potential CD4 binding sites. We first compared the CD4-binding ability of wild-type gp120 to that of gp120 mutants with deletions of 7–9 amino acid residues in the N-terminal conserved region. Second, we raised region-specific antibodies to this N-terminal conserved region and tested the ability of these antibodies to block the gp120–CD4 interaction. Results from both approaches support the proposal that the N-terminal conserved region of gp120 is involved in CD4 binding.

MATERIALS AND METHODS

Expression Vectors for gp120. To produce wild-type gp120, a 3.1-kilobase (kb) *Sal* I–*Xho* I fragment derived from HXB2 (9) was cloned into the *Xho* I site of a mammalian expression vector, pBaby (15), adopting a similar strategy used by Rekosh and coworkers (16). This construct was further modified to express the secreted form of gp120 by introducing an in-frame stop codon at the position Gln-550 of the envelope coding sequence. This construct can encode a protein containing all the coding sequence of gp120 and the first 38 amino acid residues of gp41. Two oligonucleotides, 5′-CTATTTTGTGCAACAGAGGTACAT-3′ and 5′-ATGCATGAGGATAAGCCATGTGTA-3′, were synthesized using standard cyanoethyl phosphoramidite chemistry on a MiliGen/Biosearch (Bedford, MA) model 8750 DNA synthesizer to generate deletion mutants, gp120 (δ56–62) and gp120 (δ108–116), in the same expression vector by using site-directed mutagenesis (17).

Transfection of DNA and Metabolic Labeling of gp120. To prepare radiolabeled gp120 for CD4–gp120 binding assays, 5×10^6 COS-1 cells were transfected with 10 μg of DNA by using the DEAE-dextran method (18). Two days after transfection, cells were washed twice with isotonic phosphate-buffered saline and incubated with cysteine-free RPMI 1640 medium containing 10% (vol/vol) dialyzed fetal calf serum and [^{35}S]cysteine at 100 μCi/ml (1 Ci = 37 GBq) for 8–16 hr. Cell-free supernatant was removed, centrifuged at 2000 rpm in a Beckman GH3.7 rotor for 5 min at 4°C, and frozen at −90°C until used.

Radioimmunoprecipitation and Binding of gp120 to CD4. For radioimmunoprecipitation using the goat anti-gp120 serum (10), 0.2 ml, 0.4 ml, and 0.8 ml of supernatant containing [^{35}S]cysteine-labeled gp120, gp120(δ56–62), and gp120(δ108–116), respectively, were used to adjust for different levels of gp120 expression. For CD4 binding assays, 1 ml, 2 ml, and 4 ml of radiolabeled supernatant from gp120, gp120(δ56–62), and gp120(δ108–116), respectively, were incubated with lysates prepared from 1.5×10^7 CD4-positive SupT1 cells. The resulting CD4–gp120 complexes were then precipitated by 20 μl of the ammonium sulfate-purified OKT4 monoclonal antibodies (5 mg/ml; derived from ascites fluid of mice inoculated with OKT4-producing hybridoma cells; American Type

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Abbreviation: HIV, human immunodeficiency virus.

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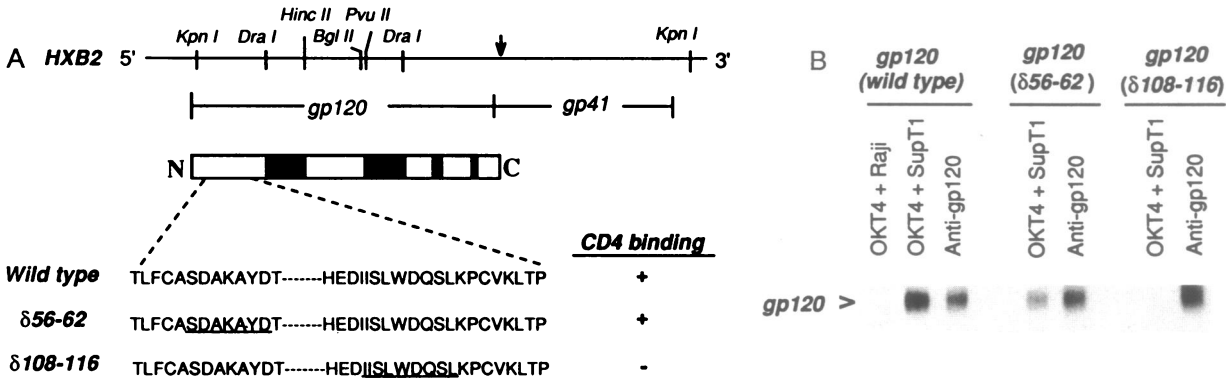

FIG. 1. Binding of mutant gp120 molecules to CD4. (*A*) Schematic diagram indicating coding sequences of three forms of secreted gp120. Selected restriction enzyme sites are indicated for orientation. Open boxes represent conserved regions of gp120 (6–8). Deleted amino acid residues are underlined. The arrow indicates the position where an in-frame stop codon was introduced. The amino acid numbering system was based on HXB2 (9) starting at the initiator methionine. The single-letter amino acid code is used. (*B*) Immunoprecipitation of gp120 by a goat anti-gp120 serum (10) and coprecipitation of CD4–gp120 complexes by the OKT4 monoclonal antibody. The goat anti-gp120 serum precipitated all three secreted forms of gp120 from supernatant of [^{35}S]cysteine-labeled COS-1 transfectants. Aliquots of [^{35}S]cysteine-labeled gp120 were used to bind CD4 molecules derived from SupT1 cells. Coprecipitation of CD4–gp120 complexes was observed with wild-type gp120 and the deletion mutant gp120(δ56–62), although binding affinity of gp120(δ56–62) appeared to be affected by the deletion. Coprecipitation of CD4–gp120 complexes was not observed with the deletion mutant, gp120(δ108–116). No gp120 was detected in this assay system when cell lysates of SupT1 cells were substituted with those of CD4-negative Raji cells.

Culture Collection CRL 8002), which were preabsorbed to 100 μl of 10% (wt/vol) protein A-Sepharose overnight at 4°C. After extensive washing, the coprecipitated gp120 was analyzed by $NaDodSO_4$/PAGE as described (19).

Preparation of gp120 Region-Specific Antisera. An envelope subclone of BH10 (20), pEnv, was constructed by cloning a 2.6-kb *Kpn* I–*Kpn* I fragment into pUC18. A 690-base-pair *Kpn* I–*Bgl* II fragment of pEnv was made blunt-ended by using the Klenow fragment of DNA polymerase I and subsequently ligated to a blunt-ended *Bam*HI–*Nco* I fragment of pXVR (21) to generate plasmid pKB. After partial digestion with *Dra* I, the linearized 4.2-kb pKB DNA was ligated with a *Xba* I linker containing an in-frame stop codon to generate plasmid pKD. This plasmid encodes a v-ras–env fusion protein that contains gp120 residues 42–129. The plasmid pDrP was constructed by inserting a 475-base-pair *Dra* I–*Pvu* II fragment from pEnv into the blunt-ended *Ava* I site in the polylinker region of a prokaryotic expression vector p806. This expression vector was constructed by cloning a modified 0.6-kb *Eco*RI–*Nco* I fragment of pXVR into the *Eco*RI site in the polylinker region of pUC18. This vector was used by us (18) to express a recombinant vpx protein of the simian immunodeficiency virus. The pDrP plasmid encodes a v-ras–env fusion protein similar to that expressed by the plasmid pKD except that it contains gp120 residues 130–288. Procedures for expression and purification of these recombinant envelope peptides and raising heterologous antisera were similar to those described previously (18). The specificity of sera was determined by Western blot analysis using an HIV strain HTLV-IIIb lysate as described (19). Preimmune and immune KD and DrP sera were diluted 1:400, and human sera were diluted 1:200.

***In Vitro* Blocking of the gp120–CD4 Interaction.** To assay the blocking activity of region-specific gp120 antisera, 10 μl of antiserum was preincubated with 10 μl of HIV-1 viral lysates in the presence of 80 μl of 1% Triton X-100-containing 0.05 M Tris·HCl/0.15 M NaCl, pH 7.2 at 4°C for 1 hr. The HIV-1 viral lysates were prepared from HTLV-IIIb-infected Molt-3 cells grown at the peak logarithmic phase. Approximately 30 ml of cell-free supernatant was centrifuged at 20,000 rpm (Beckman SW 28 rotor) for 2 hr and resuspended in 90 μl of lysis buffer containing 0.05 M Tris·HCl (pH 7.2), 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% $NaDodSO_4$ (19). The mixtures of serum and viral lysates were then added to cell lysates prepared from 1.5 × 10^7 CD4-positive SupT1 cells and incubated further at 4°C for 3 hr. The CD4–gp120 complexes were precipitated by 20 μl of ammonium sulfate-purified OKT4 antibodies (5 mg/ml), which was preabsorbed to 100 μl of 10% (wt/vol) protein A-Sepharose overnight at 4°C and then incubated an additional 20 min with 10 μl of a nonimmune goat serum to block free antibody binding sites. After extensive washing, the coprecipitated gp120 was eluted in a $NaDodSO_4$ reducing buffer and analyzed on a Western blot (19) using the same goat anti-gp120 serum described above for radioimmunoprecipitation and biotinylated anti-goat immunoglobulin and streptavidin-horseradish peroxidase complexes to visualize gp120. The stained heavy chains on blots were derived presumably from the nonimmune goat serum, and they served as controls to ensure that comparable amounts of proteins eluted from the protein A-Sepharose were loaded on the $NaDodSO_4$/polyacrylamide gels for Western blot analysis.

RESULTS

Three forms of secreted recombinant gp120 were used for CD4 binding analysis (Fig. 1*A*). In addition, an in-frame stop codon was introduced at the position corresponding to Gln-550 of the envelope coding sequence. The rationale for this truncation was to produce the secreted form of gp120 (22). The wild-type construct of gp120, secreted in cell-free culture supernatant, was specifically precipitated by sera from HIV-seropositive people and by antisera raised against gp120 recombinant peptides (23), but not by sera from the HIV-seronegative people or by preimmune sera. Fig. 1*B* shows an example of gp120 precipitated by a goat antiserum directed to gp120 from metabolically labeled culture supernatants of COS-1 transfectants. By using a similar coprecipitation assay first described by McDougal *et al.* (24), the gp120 in the culture supernatant was found to form complexes with CD4 molecules derived from CD4-positive SupT1 cells. This gp120–CD4 complex could be precipitated by the OKT4 monoclonal antibody (Fig. 1*B*) but not by the OKT4A monoclonal antibody (data not shown). The specificity of the gp120–CD4 interactions in our assay system was demonstrated by the observation that OKT4 monoclonal antibodies failed to precipitate gp120 when the lysate of CD4-positive SupT1 cells was replaced by a lysate of CD4-negative Raji cells (Fig. 1*B*).

Site-directed mutagenesis was used to generate two deletion mutants of gp120, designated gp120(δ56–62) and gp120(δ108–116). Amino acid residues at positions 56–62 and 108–116 were deleted from the N-terminal conserved region of gp120 in gp120(δ56–62) and gp120(δ108–116), respectively (Fig. 1*A*). The amino acid residues selected for deletion analysis in this N-terminal region of gp120 are highly conserved among HIV-1 isolates (6–8). Both of these deletion mutants have in-frame stop codons introduced at the same position as the secreted wild-type gp120. As shown in Fig. 1*B*, both gp120(δ56–62) and gp120(δ108–116) could be precipitated from cell-free culture supernatants of metabolically labeled COS-1 transfectants by the same goat antiserum to gp120 as described above. The CD4-binding ability of gp120(δ56–62) did not appear to be drastically altered because the gp120(δ56–62)–CD4 complex could still be coprecipitated by the OKT4 monoclonal antibody (Fig. 1*B*). However, in parallel analyses the OKT4 monoclonal antibody failed to coprecipitate gp120(δ108–116) (Fig. 1*B*), indicating that the N-terminal region of gp120 contains conserved residues that are critical for receptor binding.

The loss of CD4-binding ability with the mutant gp120(δ108–116) construct could be caused, in theory, by alterations of the overall gp120 conformation. However, as is exemplified by the mutant gp120(δ56–62), deletion of conserved gp120 residues does not necessarily cause significant alterations of overall conformation. We must therefore also consider the possibility that removal of all or part of the potential CD4 binding sites contributed to this loss of CD4-binding ability.

In the absence of x-ray crystallographic data, successful blocking of HIV–CD4 interactions by gp120 antibodies with binding specificities to a C-terminal peptide was the best evidence supporting the presence of potential CD4 binding sites in the C-terminal region of gp120 (4). We took a similar approach here to study the possibility that the N-terminal region of gp120 contains potential CD4 binding sites as well. For this purpose we raised two region-specific antisera with binding specificities to recombinant envelope peptides corresponding to different N-terminal regions of gp120. We then evaluated their ability to block HIV–CD4 interactions. One of these region-specific gp120 antisera, designated KD, was raised against a recombinant envelope peptide containing gp120 residues 42–129. The coding sequence for these residues is flanked by a *Kpn* I site and the first *Dra* I site indicated in Fig. 1*A*. The second region-specific antiserum, which served as a control for KD, was designated DrP. This antiserum was raised against a second recombinant envelope peptide containing gp120 residues 130–288. The coding sequence for these residues is flanked by the first *Dra* I restriction site and a *Pvu* II restriction site indicated in Fig. 1*A*. Both recombinant envelope peptides were expressed in *Escherichia coli* using constructs similar to a previously described vector (18). The binding specificity of these two antisera was analyzed by the Western blot technique. As shown in Fig. 2*A*, immune, but not preimmune sera reacted with gp120 prepared from an HTLV-IIIb viral lysate.

Immune KD and DrP sera and their respective preimmune sera were tested for their ability to block binding of gp120 molecules to CD4. In our assay system, HIV-1 viral lysates were first incubated with lysates prepared from 1.5×10^7 CD4-positive SupT1 cells to allow the formation of gp120–CD4 complexes. In the absence of blocking gp120 antibodies, gp120–CD4 complexes could be precipitated by OKT4 monoclonal antibodies preabsorbed to protein A-Sepharose. The CD4-bound gp120 could then be eluted from protein A-Sepharose and identified by Western blot analysis using the goat antiserum directed to gp120 described in Fig. 1*B*. No gp120 was detected when cell lysates of CD4-positive SupT1 cells were replaced by cell lysates prepared from an equiv-

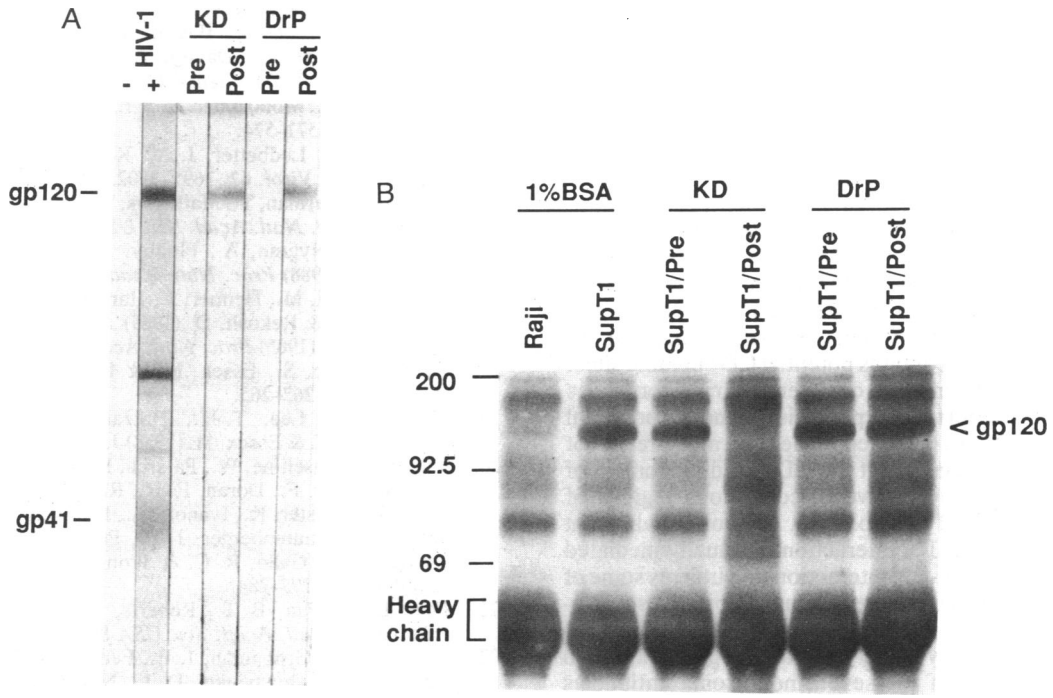

FIG. 2. (*A*) Western blot analysis of region-specific gp120 antisera. Binding reactivity of preimmune and immune (lanes Pre and Post, respectively) KD and DrP sera to HIV-1 lysates was determined by Western blot analysis, along with a positive control serum from an HIV-1-infected individual (lane +) and a negative control human serum (lane −). (*B*) Blocking of CD4–gp120 interactions by the immune KD serum. The gp120 coprecipitated by the OKT4 monoclonal antibody was detected with the Western blot technique using the same goat anti-gp120 serum described in Fig. 1. Preincubation with 1% bovine serum albumin (BSA) in Tris-buffered saline had no effect on the binding of gp120 to CD4. Preincubation with the immune KD serum, but not the preimmune KD serum, blocked CD4–gp120 interactions. Preincubation with preimmune or immune DrP sera did not block CD4–gp120 interactions. No gp120 was detected when cell lysates of the CD4-positive SupT1 cells were substituted with those of CD4-negative Raji cells. Molecular masses in kDa are shown.

alent number of CD4-negative Raji cells (Fig. 2*B*), demonstrating the specificity of CD4–gp120 interactions in this assay system. Preincubation of HIV-1 lysates with immune KD serum for 1 hr at 4°C reproducibly prevented gp120 molecules from binding CD4 (Fig. 2*B*). Preincubation of HIV-1 lysates with preimmune KD serum did not prevent the gp120–CD4 interaction. By contrast, both preimmune and immune DrP sera repeatedly failed to block the gp120–CD4 interaction (Fig. 2*B*). The most direct interpretation of these findings is that the N-terminal region of gp120 potentially contains sites that bind to CD4.

DISCUSSION

To our knowledge, mutational analysis has been conducted in the N-terminal region of gp120 in three other studies (11, 25, 26). The study by Kowalski *et al.* (11) introduced three separate in-frame insertions into the same N-terminal region that we studied here and found no effects on CD4 binding. However, since none of the insertions was introduced within the very sequences we deleted, it remains possible that CD4-binding ability was unaltered. In the study by Dowbenko *et al.* (25), a deletion mutant lacking the first 164 N-terminal amino acid residues of gp120 (up to the *Hin*cII site, indicated in Fig. 1*A*) was found to lack CD4-binding ability. This loss of CD4-binding ability was attributed to disruption of tertiary structure rather than the loss of binding sites *per se*, because two monoclonal antibodies directed to an N-terminal region of gp120 failed to block HIV–CD4 interactions. Considering that 6 of the 18 highly conserved cysteine residues (Cys-54, Cys-74, Cys-119, Cys-126, Cys-131, and Cys-157), and 7 potential N-linked glycosylation sites were removed from the mature gp120 molecule in this deletion mutant, it was perhaps correct to conclude that the overall conformation of gp120 had indeed been altered. Nevertheless, the failure of the two monoclonal antibodies to block CD4–gp120 interactions was insufficient to rule out the possibility that the N-terminal region of gp120 may contain potential CD4-binding sites. Most recently, Cordonnier *et al.* (26) reported that deletion of amino acid residues 38–46 or 82–95 of an unprocessed gp160 molecule affected CD4 binding. However, on the basis of the observations made by Nygren *et al.* (14), the diminished CD4-binding ability of these mutants was attributed to conformational changes rather than to deletion of binding sites for the CD4. Because the study by Nygren *et al.* (14) did not rule out direct participation by an N-terminal 25-kDa gp120 fragment in CD4 binding (this fragment overlaps the region we deleted), it appears premature to reject the explanation that the N-terminal region of gp120 contains potential CD4 binding sites.

Results obtained from two complementary approaches used in this study support our hypothesis that not all potential binding sites for CD4 are localized in the C-terminal region of gp120. Our findings suggest that the CD4-binding domain of HIV-1 may involve at least two discontinuous regions of gp120 at opposite ends of the molecule. It is conceivable that the high-affinity CD4–gp120 interaction is actually mediated by a cooperative multiple-site interaction that utilizes some of the 18 highly conserved cysteine residues to bring discontinuous residues into close proximity. This model of HIV–CD4 interaction, if proven by x-ray crystallography, may also explain why only two of the seven monoclonal antibodies with binding specificity to a synthetic gp120 peptide corresponding to the C-terminal CD4 binding site could achieve 50% inhibition of virus infectivity even at a concentration of greater than 10 μg/ml (27). According to this model, then, vaccine development and immunotherapeutic strategies that employ gp120 fragments should be reevaluated to consider how to simultaneously block all CD4-binding sites. Such approaches may be more likely to generate antibodies with a cooperative binding affinity greater than that of HIV–CD4 interaction, thus preventing HIV-1 infections more effectively.

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