Brief Definitive Report

Construction of A Binding Site for Human Immunodeficiency Virus Type 1 gp120 in Rat CD4

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Summary

The human immunodeficiency virus (HIV-1) infects T lymphocytes via an interaction between the virus envelope glycoprotein gp120 and the CD4 antigen of T helper cells. Previous studies demonstrated that mutations in various regions of CD4 domain 1 lead to the loss of gp120 binding. In the present study the gp120 binding site was constructed in rat CD4 by replacing rat with human CD4 sequence. A series of mutants was constructed the best of which bound gp120 with an affinity only twofold less than that of human CD4. The data indicate that the gp120 binding site of human CD4 is constituted by residues 33–58 of domain 1.

CD4 on T lymphocytes is the target for infection with HIV-1 (1) via binding of the viral glycoprotein gp120 (2). CD4 contains four Ig-related domains with domain 1 resembling the variable domain of an Ig light chain (3, 4). Domain 1 of CD4 is sufficient for high-affinity binding of gp120 (5, 6), and mutation experiments resulting in the loss of gp120 binding have been undertaken in an attempt to map the gp120 binding site. The most informative studies are those where single amino acids were changed and initially CD4 residues 42–49 were suggested to encompass the gp120 site (7). Another study identified a similar region but also raised the possibility that sequence in domain 2 is important for gp120 binding (8). Later studies showed that mutation at residue 55 destroyed gp120 binding (6) and that mutations outside the 42–55 region also affected binding, namely mutations at residues 58, 59, 67, and 68 in one study (9), and at residues 29, 59–64, 77–81, and 85 in another (10). In considering these data and the three-dimensional structure (11, 12), Ryu et al. (11) focused on the region 41–59 as being important for gp120 binding, with changes at residues 77, 81, and 85 also requiring consideration.

To complement the negative mutation data, we have constructed a gp120 binding site in rat CD4 by replacing rat with human CD4 sequence.

Materials and Methods

Mutagenesis of Rat CD4. Rat CD4 cDNA (4) was subcloned as a BamHI fragment into M13mp19 for mutagenesis using a commercial kit (Amersham International, Amersham, Bucks, UK). To introduce human CD4 sequences as shown in Fig. 1 *C*, oligonucleotide sequences were taken from the human CD4 cDNA sequence (3) and flanked with 12 bases of the rat CD4 sequence to ensure correct binding of the oligonucleotides to the template. The mutants were sequenced and the BalI fragment containing the coding sequence of mutants 2–5 was subcloned into the expression vector CDM8 (13, 14). Alternatively, PCR was used to generate constructs encoding soluble forms of the mutants as described (15).

Immunofluorescence Analysis of gp120 and Antibody Binding. Mouse mAbs and gp120: mAb OX65 reacts with domains 3 and 4 of rat CD4 (unpublished data), and mAbs 6.3G4 and 7.3F11 bind domain 1 of human CD4 (16). mAb 108 is directed against gp120 (D. Healey, unpublished data). The gp120 of HTLV-III_{3B} type was expressed in Chinese hamster ovary (CHO) cells and supplied by the MRC AIDS Directed Programme (London, UK). COS cells (4.5×10^6) were transfected with the CDM8 vectors as described (13, 14) and analyzed for gp120 or antibody binding by fluorescence-activated cell sorting (17).

Inhibition of ^{125}I-labeled Human Soluble (s)CD4 Binding to gp120. 50 µl of rabbit anti-mouse (RAM) IgG antibody at 50 µg/ml was added to the wells of a Falcon 3911 plate (Becton Dickinson and Co., Oxnard, CA) and left for 1 h at room temperature. The wells were blocked with 0.5% BSA, washed, and then 50 µl of anti-gp120 mAb 108 (tissue culture supernatant) was added to the wells for incubation overnight at 4°C. The plates were washed and 100 µl of CHO cell tissue culture supernatant containing soluble gp120 at 300 ng/ml was added to each well for incubation at 4°C for 5 h. After washing the plates 20 µl of 300–320 ng/ml ^{125}I human sCD4 (10–15 µCi/µg) was added to each well in the presence of serial dilutions in PBS, 2% BSA of unlabeled human sCD4, rat sCD4, or fivefold-concentrated tissue culture supernatants from CHO cells expressing the various soluble mutants. After overnight incubation at 4°C, the plates were washed and the radioactivity bound was measured by gamma counting.

Inhibition of ^{125}I-labeled Human sCD4 Binding to mAb 6.3G4. The assay was as for binding to gp120 except that the anti-CD4

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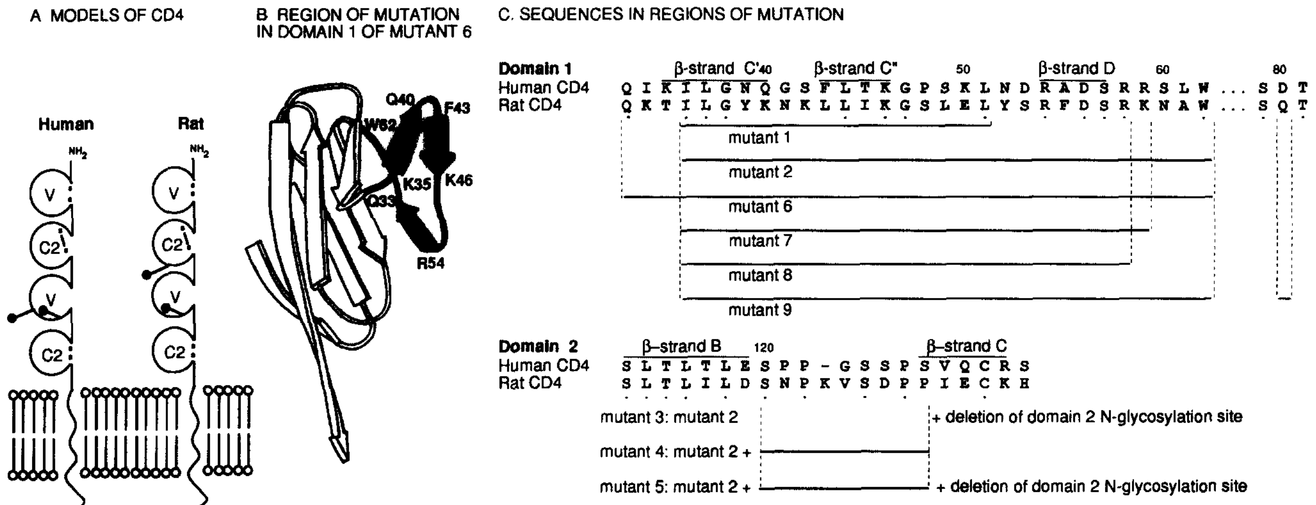

Figure 1. (*A*) Models for human and rat CD4. Glycosylation sites are shown (⫯). (*B*) The structure of CD4 domain 1 as in references 11 and 12. The filled region shows the segment of mutant 6 that is identical to human CD4. (*C*) Human and rat CD4 sequences (3, 4) in domains 1 and 2 that were exchanged in the mutants are underlined, with β strands assigned as in references 11 and 12. The dots under the rat sequence mark identical residues in the two species.

mAb 6.3G4 was bound to RAM IgG antibody on the plate and the ^{125}I human sCD4 was added at 160–200 ng/ml.

Results and Discussion

Models for human and rat CD4 are shown in Fig. 1 *A*. The level of sequence identity between the two species is 54% in the extracellular part and 50% in domain 1. A model for CD4 domain 1 is shown in Fig. 1 *B* with the region encompassed by mutant 6, which gave the best binding to gp120, shown in black. The sequences in the regions of mutation are shown in Fig. 1 *C*.

Mutant 1 containing human residues 36–51 incorporates the region first proposed for the gp120 site (7). In mutant 2 this region was extended to residue 62 on the basis of the study by Arthos et al. (6) and this mutant was then used

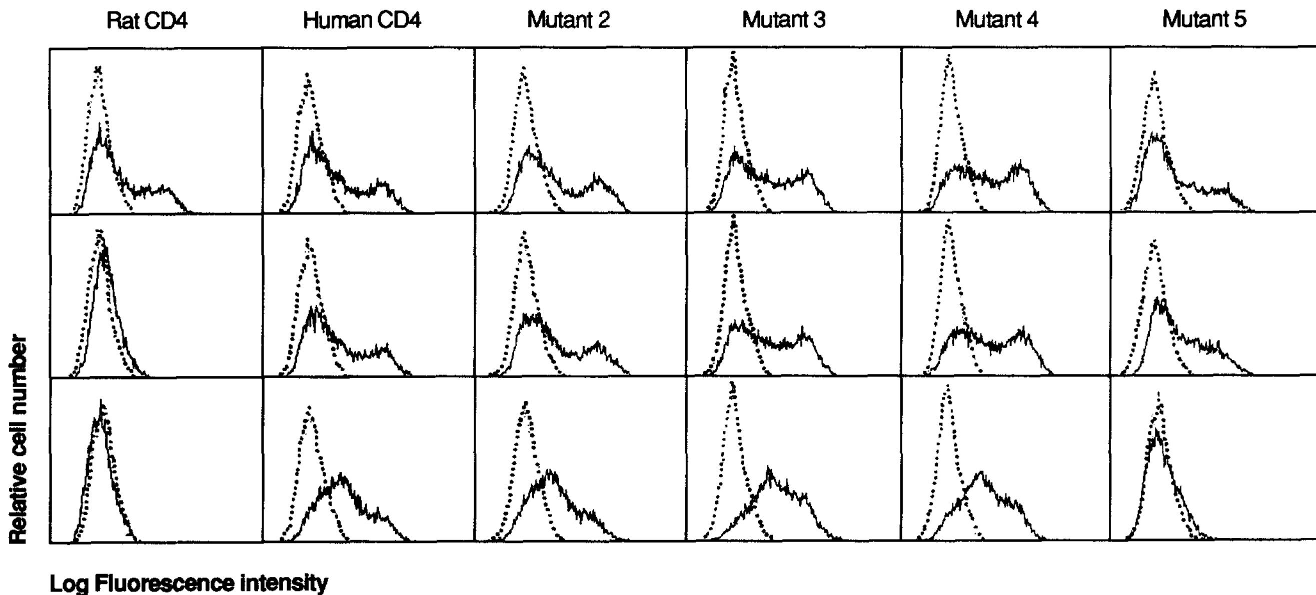

Figure 2. Binding of antibodies and gp120 to rat, human, and mutant rat CD4. COS cells were transiently transfected with CDM8 containing (13, 14) the genes for rat, human, and mutant CD4, and then analyzed by fluorescence-activated cell sorting. (*Top*) Heavy lines represent cells incubated with the anti–rat CD4 mAb OX65, except for cells expressing human CD4 that were labeled with the anti–human CD4 mAb 7.3F11 (16). Dotted lines represent cells incubated with a control mAb, OX21, that recognizes human complement C3b inactivator. (*Middle*) Heavy lines show labeling of cells with the 6.3G4 anti–human CD4 mAb (16); dotted lines represent results with the mAb OX21. (*Bottom*) Heavy lines represent cells incubated with gp120 before incubation with the anti-gp120 mAb 108; dotted lines represent cells incubated with the anti-gp120 mAb 108 only.

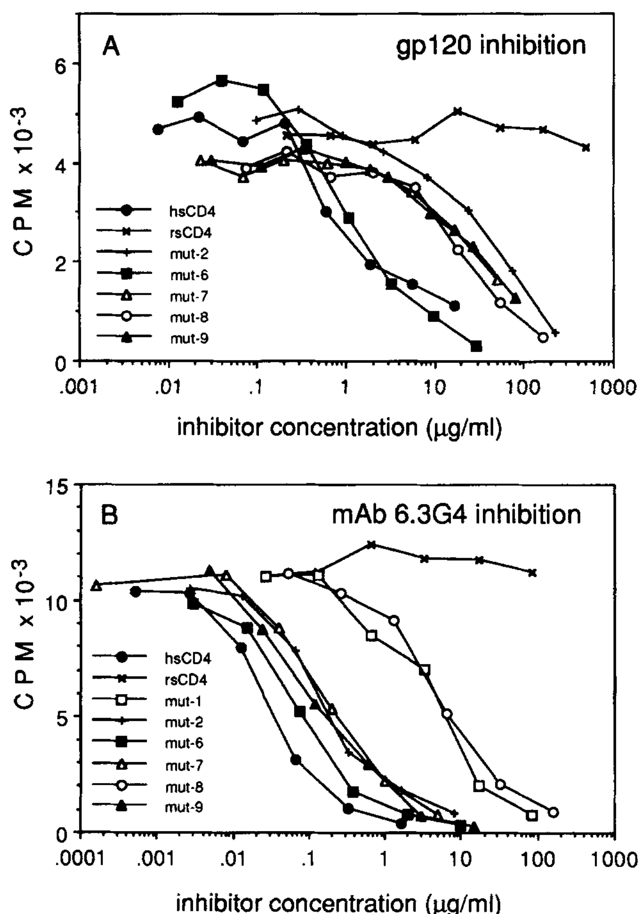

Figure 3. Mutant CD4 inhibition of ^{125}I human sCD4 binding to gp120 (*A*) or the mAb 6.3G4 (*B*). The wells of a plate coated with RAM were incubated successively with the anti-gp120 mAb 108 and then recombinant soluble HIV-1 gp120, or with the mAb 6.3G4 alone. The binding of ^{125}I-labeled human sCD4 to gp120 or the mAb was measured in the presence of serial dilutions of either human sCD4 (hsCD4), rat sCD4 (rsCD4), or soluble forms of the various mutants. The counts shown are the averages of triplicate assays and are from representative experiments.

as the basis for the subsequent mutants. In mutant 3 the *N*-glycosylation site in domain 2 of rat CD4 was removed from mutant 2, and in mutant 4 changes in the region of domain 2 postulated to be important in gp120 binding (8) were added. Mutant 5 incorporates the two changes in mutants 3 and 4. In mutants 6–9 mutant 2 was extended to Gln-33, shortened to Arg-59 or to Arg-58, or mutated at position 80 from Gln to Asp, respectively.

In initial experiments gp120 did not bind to HeLa cells expressing mutant 1. Mutants 2–5 were therefore prepared and transiently expressed in COS cells. Expression was detected for rat CD4 and mutated CD4 by labeling with the OX65 mAb that binds the COOH-terminal half of rat CD4 (unpublished data) and for human CD4 by labeling with the anti-human CD4 mAb 7.3F11 (16) (Fig. 2). In addition, human CD4 and the mutants were labeled with the 6.3G4 mAb that reacts with CD4 domain 1 (16). The binding of gp120 from HIV-1 was seen with human but not rat CD4, and mutants 2–4 were also clearly labeled with gp120. Mutant 5 was negative for gp120 binding, even though the 6.3G4 mAb bound to mutant 5.

These data indicated that the changes made in mutant 2 were sufficient to generate a gp120 binding site in rat CD4. The additional changes in mutants 3 and 4 appeared to have no effect, although these mutants were not characterized further. To determine the affinity of mutant 2 for gp120, the protein was expressed in a soluble form (15) and used in an inhibition assay in which the binding of ^{125}I human sCD4 to immobilized gp120 was measured. This assay avoids any possibility of multivalent interactions. Fig. 3 *A* shows representative data indicating that both human sCD4 and mutant 2 inhibit the binding of ^{125}I human sCD4. From eight determinations, the relative affinity of mutant 2 for gp120 was determined to be 60–70-fold less than that of human CD4. It is known that changes in one amino acid can lead to a complete loss of gp120 binding (reviewed in references 11 and 12) and therefore it seems likely that the region encompassing residues 36–62 is central to the binding site for gp120.

However, the binding site for gp120 was not fully constituted by residues 36–62, and therefore additional mutants were constructed. Inspection of the x-ray crystallographic structure of human CD4 suggested that nonconservative substitutions present in mutant 2 at the C, C′ turn could affect the binding site. Specifically, Ile-34 and Lys-35 are substituted by Lys and Thr residues, respectively (Fig. 1). Therefore, in mutant 6 the humanized region was extended back to the conserved Gln at position 33 and this mutant was shown to have an affinity for gp120 only twofold less than that of human CD4 (Fig. 3 *A*). This indicates that the C, C′ turn has a key role in forming the binding site for gp120, but it is not yet clear which of the two substitutions is responsible for the improved binding. In other studies, Ile-34 has been mutated to Arg (6, 8) and Lys-35 mutated to Glu (7) and Ala (10) but these mutations had little or no effect on gp120 binding. The present study suggests that some substitutions in this region cannot be tolerated. The Lys at position 34 might specifically interfere with hydrophobic packing or Thr at position 35 might form inappropriate hydrogen bonds in mutant 2 that disrupt the C′, C″ loop.

In mutants 7 and 8, rat CD4 residues were restored at positions 60–61 and 59–61. This was done because inspection of the CD4 structure indicated that the side chains of the human CD4 residues Arg-59/Ser-60/Leu-61 point away from the C′, C″ loop and thus these residues might not be essential for binding. Moreover, mutagenesis experiments have shown that Ser-60 can be mutated to Arg (7, 9) or Ala (10), and Leu-61 mutated to Arg (9) without disrupting gp120 binding, and that the substitution of Arg-59 with Gly (9) or Ala (10) only partially disrupts binding. Mutant 7 was marginally better at inhibiting gp120 binding than mutant 2, and mutant 8, with the addition of the Lys-59/Arg interchange, reproducibly bound gp120 with a three-fold-higher affinity than mutant 2 (Fig. 3 *A*). Studies with the 6.3G4 antibody (Fig. 3 *B*) showed that changes in this region can affect binding reactions since mutant 8 with the Arg-59/Lys interchange binds about 40 times less well to the 6.3G4 anti-

body than do mutants 2 or 7. Taken together, the gp120 and antibody binding data argue against residues 59–61 being centrally involved in the gp120 binding site but Arg-59 could be on the periphery of the site.

Mutant 9 was made because mutation analysis of human CD4 had suggested the involvement of amino acids 77–81 (10) in gp120 binding and because residue 80 differs between rat and human CD4. The rat Gln residue was mutated to Asp in mutant 9, but this had only a small beneficial effect on the binding of gp120 (Fig. 3 *A*).

In conclusion, these experiments strongly indicate that the gp120 binding site of human CD4 is constituted by residues 33–58 and highlights the importance of the C, C′ turn in forming the binding site. In previous studies residues outside this region have been mutated to disrupt gp120 binding, but the data presented here argue against the possibility that these regions are part of the binding site.

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