× PCR buffer [10 mM Tris (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM of each dinucleoside triphosphate) containing 5 μCi of [α-^{32}P]dCTP, 0.5 μM of each primer set, and 2.5 units of AmpliTaq Gold (Perkin-Elmer). The amount of each mimic DNA added to the reaction to obtain similar amplification between mimic and target [usually 0.1 to 0.5 amol (10^{-18} mol) of mimic DNA was added depending on transfection efficiency] and the number of amplification cycles required to avoid overamplification were determined by initial amplification reactions. Samples were amplified by repeated cycles (usually 30 cycles) at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. A portion (10 μl) of each reaction mixture was subjected to electrophoresis on 5% polyacrylamide gels, dried, and visualized by autoradiography. The following primers were used: 5′-TCTGACTGACCGCGTTACTC-3′ and 5′-TTCT-TCTAGACATAGAAGATGTTTCAGTTC-3′ for IL-2; 5′-TCTGACTGACCGCGTTACTC-3′ and 5′-CACT-GCATA CGACGATTCTGT-3′ for LUC; and 5′-TCT-GACTGACCGCGTTACTC-3′ and 5′-TGCAAGGC-GATTAAGTTGGG-3′ for GAL.

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26. Various regions of IL-2 cDNA were deleted. Del 1 was constructed by creating an Eco RV site at codon 4 and codon 5 of IL-2 with PCR-based method to obtain pβACT-IL2RV. The Hind III to Eco RV fragment was replaced with an oligonucleotide that restored the start codon of IL-2. Del 2 was constructed by removing a Eco RV to Afl II fragment from pβACT-IL2RV and replacing it with a PCR fragment digested with Apa LI/Afl II, in which nt 67 to 114 were removed. Del 3 was constructed by subcloning two PCR fragments—nt 1 to 130, which had been digested with Hind III and Bgl II, and nt 200 to 350, which had been digested with Bgl II and Afl II—between the Hind III and Afl II sites of pβACT-IL2. Del 4 and Del 5 were constructed by digesting pβACT-IL2 with Afl II and Stu I, and with Stu I and Sty I, respectively, and then religating with T4 DNA ligase. Del 6 was constructed by removing a Sty I-Kpn I fragment pβACT-IL2 and replacing it with an oligonucleotide containing the polyadenylation signal of IL-2. None of the IL-2 mutants altered the reading frame.
27. The entire 3′ UTR, including the polyadenylation signal of human *c-fos* or GM-CSF amplified by RT-PCR, and a chemically synthesized double-stranded oligonucleotide containing two nonamers, UUAUUUAUU-gauccUUAUUUAUU, and a polyadenylation signal were subcloned between the Stu I and Kpn I sites of pβACT-IL2. CAT reporters were constructed by subcloning the entire coding region of the *CAT* gene, amplified by PCR, between the Hind III and Stu I sites of pβACT-IL2 to obtain CAT-3′ UTR (IL2) or between the Bgl II and Stu I sites of Del 3 to obtain 5′ UTR (IL2)–CAT–3′ UTR (IL2), in which the reading frame of CAT is in-frame with that of IL-2.
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29. Jurkat whole-cell extract (50 μg of protein) was resolved by SDS-PAGE and transferred to an Immobilon P membrane (Millipore). After blocking, the filter was incubated with an antibody specific to phospho-Ser^{63}-c-Jun (KM-1, Santa Cruz Biotechnology), and the antibody-antigen complexes were visualized by enhanced chemiluminescence (Amersham). The membrane was stripped and reprobed with an antibody to c-Jun (N-G, Santa Cruz Biotechnology).
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A Conserved HIV gp120 Glycoprotein Structure Involved in Chemokine Receptor Binding

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The entry of primate immunodeficiency viruses into target cells depends on a sequential interaction of the gp120 envelope glycoprotein with the cellular receptors, CD4 and members of the chemokine receptor family. The gp120 third variable (V3) loop has been implicated in chemokine receptor binding, but the use of the CCR5 chemokine receptor by diverse primate immunodeficiency viruses suggests the involvement of an additional, conserved gp120 element. Through the use of gp120 mutants, a highly conserved gp120 structure was shown to be critical for CCR5 binding. This structure is located adjacent to the V3 loop and contains neutralization epitopes induced by CD4 binding. This conserved element may be a useful target for pharmacologic or prophylactic intervention in human immunodeficiency virus (HIV) infections.

Most naturally occurring primate immunodeficiency viruses [HIV and simian immunodeficiency virus (SIV)] bind the β-chemokine receptor CCR5 as an obligate step in virus entry into target cells (*1*, *2*). The gp120 glycoproteins of primary, macrophage-tropic HIV-1 strains have been shown to bind specifically to cells expressing CCR5 (*3*, *4*). Inhibiting this binding constitutes an attractive means of intervening in HIV-1 infection, but the gp120 glycoprotein is a challenging target because of its conformational flexibility and variability. Incubation with soluble CD4 (sCD4) results in a 100- to 1000-fold increase in the affinity of HIV-1 gp120 for CCR5 (*3*), indicating that the CCR5-binding site on HIV-1 gp120 is fully formed or exposed only after CD4 binding. Efficient CCR5 binding is dependent on the presence of the V3 variable loop of gp120 (*3*), and the sequence of the V3 loop influences the specific chemokine receptors used by different primate immunodeficiency viruses (*2*). In contrast, the gp120 V1 and V2 variable loops and NH_2- and COOH-termini were shown to be dispensable for high-affinity binding to CCR5 (*3*). No significant CCR5 binding has been observed for gp120 glycoproteins derived from laboratory-adapted HIV-1 isolates, which do not use CCR5 as a coreceptor (*3*, *4*).

Specific groups of HIV-1–neutralizing antibodies directed against the gp120 V3 loop or CD4-induced (CD4i) epitopes are able to block the binding of gp120-sCD4 complexes to CCR5-expressing cells (*3*, *4*). The CD4i epitopes are conserved, discontinuous gp120 structures that are better exposed after CD4 binding (*5*). Mutagenic analysis suggested that elements of the conserved stem of the V1/V2 stem-loop and of the fourth conserved region of gp120 make up the CD4i epitopes (*5*). Here we tested the hypothesis that conserved gp120 residues near or within the CD4i epitopes are critical for CCR5 binding.

We developed an assay to assess the CCR5-binding ability of a panel of HIV-1 gp120 glycoprotein mutants. The mutants were created by the introduction of single amino acid changes in gp120 residues near or within regions previously shown to be

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important for the integrity of the CD4i epitopes (5). Structural information (6) on the gp120 epitope recognized by a CD4i-directed antibody, 17b, was used to guide the mutagenesis. The wtΔ glycoprotein, which lacks the V1 and V2 variable loops and the NH_2-terminus and is derived from the YU2 primary macrophage-tropic (R5) HIV-1 isolate (7), was the starting point for our studies (Fig. 1). This protein was chosen because it had been shown to bind CD4 and CCR5 with high affinity (3, 8, 9). Furthermore, the use of this protein minimized the opportunities for indirect effects of gp120 amino acid changes on CCR5 binding [for example, by repositioning the V1 and V2 loops, which can mask CD4i epitopes (9)]. Metabolically labeled wtΔ and mutant derivatives were produced in 293T cells and incubated with mouse L1.2 cells stably expressing human CCR5 (3), in either the absence or presence of sCD4. The cells were washed and lysed, and bound gp120 protein was detected by precipitation with a mixture of sera from HIV-1–infected individuals (10).

The wtΔ protein efficiently bound to the L1.2-CCR5 cells in the presence of sCD4 (Fig. 2, A and B). Binding was significantly reduced when sCD4 was not present in the assay. Binding of the wtΔ protein to the L1.2-CCR5 cells was inhibited by preincubation of the wtΔ protein with the 17b antibody. Binding was also inhibited by incubation of the L1.2-CCR5 cells with the 2D7 antibody to CCR5 (11) or with the CCR5 ligand MIP-1β (12). The C11 antibody, which is directed against a gp120 region dispensable for CCR5 binding (3), did not block the binding of the wtΔ protein to the L1.2-CCR5 cells (13). An irrelevant chemokine, SDF-1α, did not efficiently block wtΔ binding (13). The wtΔ protein did not bind appreciably to the parental L1.2 cells not expressing CCR5, even in the presence of sCD4. These results suggest that the wtΔ protein binds CCR5 in a specific, CD4-dependent manner.

Changes in several gp120 amino acids resulted in reductions in the ability of the protein to bind to L1.2-CCR5 cells in the presence of sCD4 (Table 1 and Fig. 2C). In some cases (257 T/D, 370 E/Q, and 383 F/S), the attenuated CD4-binding ability of the mutant proteins could account for the observed reduction in binding to the L1.2-CCR5 cells. In most cases, however, the mutant proteins that were deficient in CCR5 binding still bound sCD4 and at least one monoclonal antibody that recognizes a discontinuous gp120 epitope (5, 14). As expected, some of the introduced amino acid changes decreased recognition by the 17b antibody. Two of the gp120 amino acid changes (437 P/A and 442 Q/L) resulted in an increase in CCR5 binding compared with the wtΔ protein, even though CD4 binding was not significantly increased. In the absence of sCD4, the 437 P/A and 442 Q/L envelope glycoprotein mutants bound to the L1.2-CCR5 cells slightly better than the other mutants and the wtΔ protein, which exhibited very low levels of binding [Fig. 2A and (13)].

Recently, the structure of an HIV-1 gp120 core crystallized in a ternary complex with two-domain CD4 and the 17b Fab was solved (6). The gp120 core is composed of an inner domain, an outer domain, and a "bridging sheet" (Fig. 3A). The bridging sheet is a four-stranded, antiparallel β sheet that includes the V1/V2 stem and strands (β20 and β21) derived from the fourth conserved gp120 region. CD4 contacts gp120 residues in the outer domain and the bridging sheet (6). The gp120 residues implicated by our study in CCR5 binding are located near or within the bridging sheet (Fig 3, A and B). The bridging sheet is predicted to face the target cell after the envelope glycoproteins bind CD4 (6). Even more than the CD4-binding site, the gp120 region implicated in CCR5 binding is highly conserved among primate immunodeficiency viruses; this conservation contrasts with the variability that characterizes most of the gp120 surface thought to be exposed on the assembled envelope glycoprotein complex (Fig. 3C) (6). The CD4i epitope for the 17b antibody is located near or within the bridging sheet (6), consistent with the ability of the antibody to block CCR5 binding (3, 4). All of the individual gp120 residues in which changes disrupted recognition by 17b antibody (Fig. 3D) are located close to the gp120-17b interface in the crystallized complex (Table 1). The binding of another antibody, CG10, which disrupts gp120-CCR5 interaction (3) and competes with 17b antibody for gp120 binding (15), is also affected by changes in amino acid residues within or near the bridging sheet (Fig. 3E). The position and orientation of the V3 base in the structure (6), in conjunction with a number of mutagenic and antibody competition studies (16), suggest that the gp120 V3 loop resides proximal to the region implicated in CCR5 binding (Fig. 3A). For example, the binding

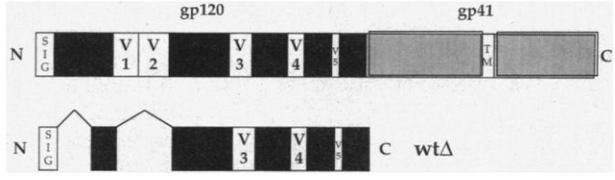

Fig. 1. The HIV-1 YU2 gp120 derivative used in the binding assay. The wild-type gp120 and gp41 envelope glycoproteins are shown in the upper figure. Conserved (black) and variable (white) regions (27) are indicated. The wtΔ protein, which is derived from the primary macrophage-tropic YU2 HIV-1 isolate (7), is shown in the lower figure. The NH_2-terminal (N) and V1 and V2 deletions correspond to those previously described for the HXBc2 gp120 mutants Δ82 and Δ128-194, respectively (8, 9). SIG, signal peptide; C, COOH-terminal; TM, transmembrane section of gp41.

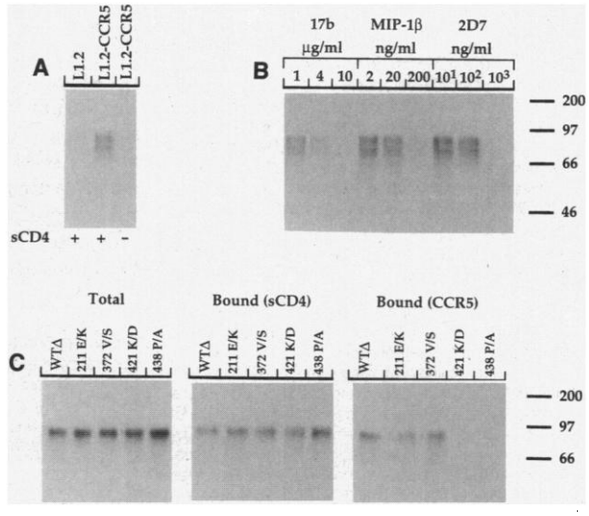

Fig. 2. The gp120-CCR5 binding assay. (**A**) The radioactively labeled wtΔ protein was incubated either with the parental L1.2 cells or with the L1.2–CCR5 cells. Incubations were carried out either in the absence or presence of sCD4 (100 nM). The wtΔ protein bound to the cells is shown. The two bands represent different glycoforms of gp120. (**B**) The wtΔ protein was incubated with both sCD4 and the 17b antibody at the indicated concentrations before addition to the L1.2-CCR5 cells. The L1.2-CCR5 cells were incubated with 2D7 antibody to CCR5 or MIP-1β at the indicated concentrations before incubation with wtΔ-sCD4 complexes. The wtΔ protein bound to the cells is shown. (**C**) The amount of radioactively labeled wtΔ or selected mutant envelope glycoproteins precipitated by a mixture of HIV-1–infected patient sera (Total), precipitated by sCD4 and an antibody to CD4 [Bound (sCD4)], or bound to L1.2-CCR5 cells [Bound (CCR5)] is shown. Molecular sizes in (B) and (C) are in kilodaltons.

of both CG10 and CD4i antibodies to gp120 can be disrupted by some V3 changes (*5*, *15*). Furthermore, several V3-directed antibodies compete with CD4i antibodies for gp120 binding (*16*).

Our observations suggest that the CCR5-binding site is likely composed of conserved gp120 elements near or within the bridging sheet and V3 loop residues. The latter might include more conserved structures (such as the aromatic or hydrophobic residue at position 317, altered in this study) as well as more variable structures (*17*) that determine the specific chemokine receptor used. Some of the gp120 residues identified in this and previous studies (*17*) as determinants of chemokine receptor use could modulate the interaction of the V3 loop and elements near the bridging sheet. For example, studies of HIV-1 revertants (*16*) suggested a functional interaction of gp120 residue 440, shown here to influence CCR5 binding, with the V3 loop.

A subset of the gp120 residues in or near the bridging sheet likely contacts CCR5 directly. Most of the gp120 residues implicated in CCR5 binding exhibit reasonable solvent accessibility in the free gp120 core (Table 1), consistent with this possibility. The gp120 surface implicated in CCR5 binding is highly basic (*6*), potentially favoring interactions with the acidic CCR5 NH_2-terminus, which has been shown to be important for gp120 binding (*18*, *19*). Additional, hydrophobic interactions, similar to those seen for gp120-17b binding (*6*), may also contribute to the gp120-CCR5 interaction.

The exposure or formation (or both) of the CCR5-binding site of HIV-1 gp120 glycoproteins is dependent on interaction with CD4 (*3*, *4*). CD4 binding has been shown to reposition the V1 and V2 variable loops and thus expose the CD4i epitopes (*9*), which overlap the CCR5-binding region (*3*, *4*). However, because a gp120 glycoprotein lacking the V1 and V2 variable loops also exhibits CD4-dependent CCR5 binding (*3*), the interaction with CD4 must cause other conformational changes in gp120 related to the CCR5-binding site. Our results, which highlight the proximity of the two receptor-binding sites on gp120, provide likely explanations for the induction of such conformational changes. First, the V1/V2 stem, which is one of the components of the bridging sheet, contacts CD4 (*6*). Thus, CD4 binding, which appears to distort the V1/V2 stem, may reposition this structure and allow the formation of the β-sheet important for CCR5 binding. In this respect, we note that a substitution of aspartic acid for threonine-123, which is located in the V1/V2 stem and contacts CD4, significantly decreases CCR5 binding. This substitution may disrupt CD4-induced conformational changes in the V1/V2 stem required for CCR5 binding.

Second, the CD4-bound conformation of gp120 exhibits a cavity (the "Phe-43" cavity) within the gp120 interior (*6*). This cavity contacts the gp120 inner and outer domains as well as the bridging sheet and likely forms as a result of interdomain conformational changes in gp120 induced by

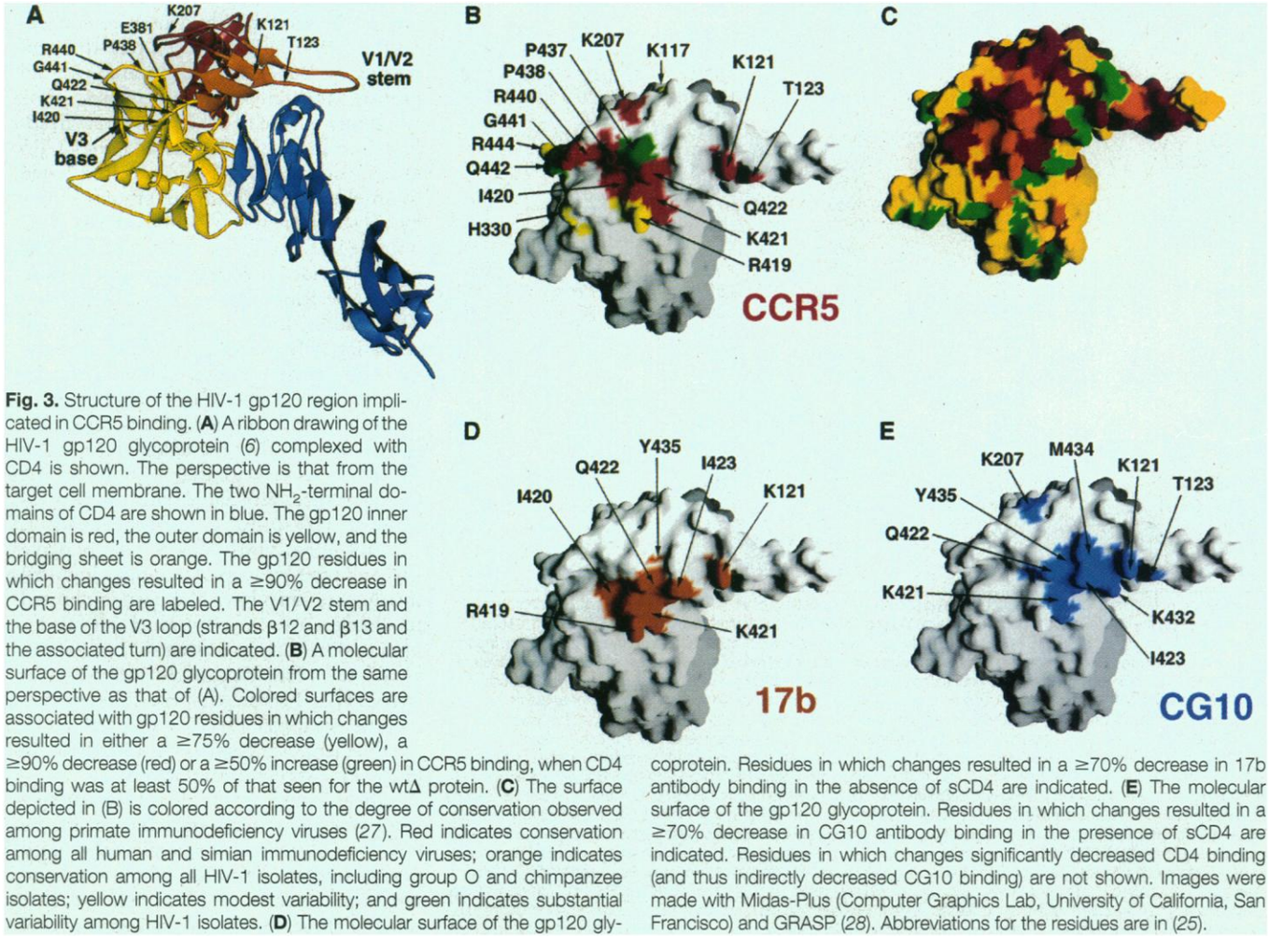

Fig. 3. Structure of the HIV-1 gp120 region implicated in CCR5 binding. (**A**) A ribbon drawing of the HIV-1 gp120 glycoprotein (*6*) complexed with CD4 is shown. The perspective is that from the target cell membrane. The two NH_2-terminal domains of CD4 are shown in blue. The gp120 inner domain is red, the outer domain is yellow, and the bridging sheet is orange. The gp120 residues in which changes resulted in a ≥90% decrease in CCR5 binding are labeled. The V1/V2 stem and the base of the V3 loop (strands β12 and β13 and the associated turn) are indicated. (**B**) A molecular surface of the gp120 glycoprotein from the same perspective as that of (A). Colored surfaces are associated with gp120 residues in which changes resulted in either a ≥75% decrease (yellow), a ≥90% decrease (red) or a ≥50% increase (green) in CCR5 binding, when CD4 binding was at least 50% of that seen for the wtΔ protein. (**C**) The surface depicted in (B) is colored according to the degree of conservation observed among primate immunodeficiency viruses (*27*). Red indicates conservation among all human and simian immunodeficiency viruses; orange indicates conservation among all HIV-1 isolates, including group O and chimpanzee isolates; yellow indicates modest variability; and green indicates substantial variability among HIV-1 isolates. (**D**) The molecular surface of the gp120 glycoprotein. Residues in which changes resulted in a ≥70% decrease in 17b antibody binding in the absence of sCD4 are indicated. (**E**) The molecular surface of the gp120 glycoprotein. Residues in which changes resulted in a ≥70% decrease in CG10 antibody binding in the presence of sCD4 are indicated. Residues in which changes significantly decreased CD4 binding (and thus indirectly decreased CG10 binding) are not shown. Images were made with Midas-Plus (Computer Graphics Lab, University of California, San Francisco) and GRASP (*28*). Abbreviations for the residues are in (*25*).

Table 1. Phenotypes of HIV-1 YU2 gp120 mutants. The ability of the wtΔ and mutant glycoproteins to bind CCR5 expressed on L1.2 cells was determined (*10*). The recognition of the wtΔ and mutant glycoproteins by sCD4 and monoclonal antibodies that recognize discontinuous gp120 epitopes (*5, 14*) was determined (*10*). All values reported are relative to those seen for the wtΔ protein. Values represent the average of at least two independent experiments and exhibit less than 30% variation from the value shown.

Protein (FSA)*	CCR5 binding†	Ligand binding‡			
		sCD4	17b	CG10	F105
wtΔ	1.00	1.00	1.00	1.00	1.00
107 D/R	1.02	1.02	0.97	1.11	1.14
114 Q/L	1.22	0.79	0.73	0.71	0.75
117 K/D (0.45)	0.15	0.74	0.64	0.42	0.83
121 K/D (0.57)	0.07	0.73	0.11§	0.0	0.99
122 L/S	0.98	0.84	1.07	0.18	1.11
123 T/D (0.49)	0.08	0.99§	1.06	0.0	1.25
197 N/D	1.33	1.34	0.80	0.81	1.11
199 S/L	1.50	1.32	0.94	1.03	1.04
200 V/S	0.84	0.91	1.05§	0.49	1.06
201 I/A	0.46	0.90	0.67	0.84	0.81
203 Q/L	0.68	0.85	0.88§	0.52	0.93
207 K/D (0.23)	0.0	0.85	0.46	0.13	0.98
209 S/L	1.00	1.11	0.85	1.01	1.00
210 F/S	0.65	0.81	0.81	0.85	0.74
211 E/K	0.73	1.13	1.03	1.12	1.24
257 T/D	0.05	0.0§	0.49	0.06	0.0
295 N/E	0.86	0.75	0.73	0.98	0.79
308 N/D	0.31	1.10	0.89	0.93	1.03
317 L/S	0.08	1.12	1.05	1.13	1.03
330 H/A	0.22	0.75	0.55	0.66	0.64
ΔV3 (Δ298-329)	0.0	0.80	0.08	1.27	0.93
370 E/Q	0.17	0.0§	1.04	0.12	0.0
372 V/S	0.85	1.03	1.08	1.09	0.44
373 T/D	0.48	1.12	1.10	1.16	1.10
377 N/E (0.04)	0.22	0.71	0.52	0.65	0.60
381 E/R (0.07)	0.07	0.81	0.75	0.29	0.96
383 F/S	0.04	0.0	0.0	0.07	0.0
386 N/D	1.22	1.14	0.97	0.90	0.97
419 R/D (0.82)	0.19	0.86	0.02§	0.48	0.82
420 I/R (0.14)	0.06	0.59	0.0§	0.72	0.72
421 K/D (0.32)	0.07	0.86	0.19§	0.0	0.0
422 Q/L (0.35)	0.07	0.53	0.0§	0.20	0.55
423 I/S	0.61	0.97	0.05§	0.30	1.03
424 I/S	0.37	0.25	0.48	0.83	0.81
426 M/A	0.75	0.69§	0.69	0.72	1.11
429 E/R	1.54	1.17§	1.00	1.05	0.82
432 K/A	0.61	1.0	0.92§	0.0	1.45
434 M/A	1.22	0.90	0.65§	0.07	1.04
435 Y/S	0.21	0.33	0.22§	0.29	1.00
436 A/S	0.98	1.05	0.91	0.99	1.23
437 P/A	1.79	0.80	0.68§	0.78	0.82
438 P/A (0.28)	0.06	1.18	1.00	1.13	1.18
439 I/A	0.45	0.68	0.76	0.76	0.84
440 R/D (0.43)	0.09	1.03	1.05	1.05	1.13
441 G/V (0.91)	0.0	0.67	0.70	0.62	0.78
442 Q/L	2.00	1.11	0.74	1.05	0.83
444 R/D (0.80)	0.25	0.79	0.67	0.94	0.74
474 D/R	1.03	0.59§	0.81	0.74	0.0

*The residue number of the mutant wtΔ glycoproteins is based on the sequence of the prototypic HXBc2 gp120 glycoprotein (*27*), with 1 representing the initiator methionine. The wild-type YU2 gp120 residue is listed first, followed by the substituted residue (*25*). The fractional solvent accessibilities (FSAs) associated with gp120 residues in which changes specifically disrupted CCR5 binding are shown in parentheses. Fractional solvent accessibility was calculated as the ratio of solvent-accessible surface area for atoms of amino acid residue X in the gp120 core (without carbohydrate moieties) to the area obtained after reduction of the structure to a Gly-X-Gly tripeptide (*24*). Values cited are for side-chain atoms, except for glycine-441 where the value for all atoms is given. †The binding of the wtΔ glycoprotein to L1.2-CCR5 cells was shown to be linearly related to the concentration of wtΔ protein in the transfected 293T cell supernatants, over the range of concentrations used in these experiments (*13*). The total amount of wtΔ and mutant glycoprotein present in the 293T cell supernatants was estimated by precipitation with an excess of a mixture of sera from HIV-1–infected individuals. The amount of wtΔ and mutant glycoprotein bound to the L1.2-CCR5 cells was determined as described (*10*). The value for CCR5 binding was calculated with the following formula: CCR5 binding = (Bound mutant protein ÷ Bound wtΔ protein) × (Total wtΔ protein ÷ Total mutant protein). ‡The recognition of the wtΔ and mutant glycoproteins by sCD4 and antibodies was determined by precipitation of radioactively labeled envelope glycoproteins in transfected 293T cell supernatants as described (*10*). In parallel, the labeled envelope glycoproteins were precipitated with an excess of a mixture of sera from HIV-1–infected individuals. The value for ligand binding was calculated with the following formula: Ligand binding = (Mutant $protein_{ligand}$ ÷ wtΔ $protein_{ligand}$) × (wtΔ $protein_{serum\ mixture}$ ÷ Mutant $protein_{serum\ mixture}$). §In the sCD4 and 17b columns, the values indicate gp120 residues that exhibit decreased solvent accessibility in the presence of the two-domain sCD4 or 17b Fab, respectively, in the ternary complex (*6*). Changes in solvent accessibility were calculated with the MS program of M. Connolly (*26*).

CD4 binding (6). Because the bridging sheet lacks its own hydrophobic core and is thus dependent on residues contributed by both inner and outer domains (6), any shift in orientation between these domains would alter the conformation of the bridging sheet. Furthermore, CD4 binding could also alter the precise orientation of the bridging sheet with respect to the inner and outer domains, thus aligning the V3 loop and conserved gp120 elements important for CCR5 binding. To summarize, CD4 binding likely induces conformational changes within the bridging sheet as well as between this sheet and the inner and outer domains to form the high-affinity CCR5-binding site. For some primate immunodeficiency viruses, the CD4-bound conformation of gp120 must be energetically accessible in the absence of CD4 to explain the documented examples of CD4-independent chemokine receptor binding and entry (*19, 20*).

It is likely that the CCR5-binding region defined in this study is also important for the binding of simian and human immunodeficiency viruses to other chemokine receptors. The identified region exhibits one of the most highly conserved surfaces on the HIV-1 gp120 glycoprotein (6), supporting its functional importance for all primate immunodeficiency viruses. The laboratory-adapted HXBc2 envelope glycoprotein, which uses CXCR4 and not CCR5 as a coreceptor (*1, 2, 21*), can be converted to an efficient CCR5-using protein simply by substituting the V3 loop of the YU2 virus (2). Thus, all of the CCR5-binding region outside of the V3 loop must be conserved, at least between the HXBc2 and YU2 viruses. Indeed, we have shown that alteration of lysine-117, lysine-207, and glycine-441 in the HXBc2-YU2V3 chimeric protein also disrupts CCR5 binding (*13*). Consistent with the use of this region for the binding of other chemokine receptors is the observation (*20*) that a subset of the gp120 changes associated with the conversion of HIV-2 to a CD4-independent, CXCR4-using virus affects the bridging sheet and the V3 loop. Alterations in bridging sheet residues have also been implicated in changes in the tropism of HIV-1 for immortalized cell lines that do not express CCR5 (*22*). Finally, the 17b antibody neutralizes HIV-1 strains that use different chemokine receptors (*5, 15*), supporting the involvement of a common gp120 region in chemokine receptor interaction.

Chemokine receptor binding may trigger additional conformational changes in the envelope glycoprotein complex that ultimately lead to the fusion of the viral and target cell membrane. It is believed that some of these changes include exposure of

the ectodomain of the gp41 transmembrane envelope glycoprotein (23). It is interesting that the CCR5-binding region defined herein likely resides close to the trimer axis of the assembled envelope glycoprotein complex (6). Indeed, some of the gp120 residue changes that affect CCR5 binding also affect the noncovalent association of gp120 and gp41 subunits in the trimeric complex (13). These observations raise the possibility that chemokine receptor binding alters the relation between gp120 and gp41, leading to the exposure of the gp41 ectodomain and interaction with the target cell membrane.

The definition of a highly conserved gp120 structure that is important for binding to CCR5 should assist the development of pharmacologic or immunologic inhibitors of virus-receptor interactions. An understanding of the CD4-induced conformational changes in this structure may allow the targeting of such inhibitors to native or CD4-bound states of gp120.

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10. We cotransfected 293T cells with 20 μg of a plasmid expressing the wtΔ or mutant envelope glycoproteins and 2 μg of a plasmid expressing the HIV-1 Tat protein using the calcium phosphate technique. Transfected cells were washed and metabolically labeled for 16 hours with [^{35}S]cysteine (50 μCi/ml) and [^{35}S]methionine (50 μCi/ml). Labeled cell supernatants were harvested, cleared by low-speed centrifugation (200*g* for 10 min at 4°C), and stored at 4°C until used in the binding assays. For measurement of the binding of sCD4 and antibodies to the wtΔ and mutant envelope glycoproteins, different dilutions of the envelope glycoprotein–containing supernatants were precipitated to ensure that binding occurred in the linear range of the assay. For CD4 binding, the envelope glycoprotein–containing supernatants were incubated for 30 min at room temperature with a concentration of sCD4 (SmithKline Beecham) empirically determined to precipitate the wtΔ protein optimally. The envelope glycoprotein–sCD4 complexes were then precipitated with the CD4-specific antibody OKT4 (Ortho) and protein A–Sepharose (Pharmacia). For binding of the 17b and F105 antibodies, the monoclonal antibodies were incubated with protein A–Sepharose before overnight incubation with envelope glycoprotein–containing supernatants at 4°C. For binding of the CG10 antibody, envelope glycoprotein–containing supernatants were incubated with 100 nM sCD4 at room temperature for 30 min before addition of a CG10–protein G–Sepharose mixture and overnight incubation at 4°C. Immunoprecipitates were washed and run on 12.5% SDS-polyacrylamide gels, which were fixed, dried, and analyzed by autoradiography. Binding was quantified by densitometry. To measure CCR5 binding, we mixed envelope glycoprotein–containing supernatants with 100 nM sCD4 or phosphate-buffered saline (PBS) and incubated them at room temperature for 30 to 60 min. L1.2-CCR5 cells [2×10^7 cells, LeukoSite (3)] were pelleted, resuspended in 500 μl of envelope glycoprotein–containing supernatants, and rocked gently at 37°C for 1 hour. Cells were pelleted, washed twice in PBS, and lysed by the addition of NP-40 buffer (0.5 M NaCl, 10 mM tris, pH 7.5, 0.5% NP-40). Lysates were cleared (20,000*g* at 4°C for 15 min) in a microcentrifuge, and the envelope glycoproteins were precipitated overnight at 4°C by a mixture of sera from HIV-1–infected individuals and protein A–Sepharose. Sepharose pellets were washed in NP-40 buffer, boiled in SDS-containing sample buffer, and run on 12.5% SDS-polyacrylamide gels. Autoradiographed gels were quantitated with a densitometer.
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25. Amino acid abbreviations are as follows: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; and Y, tyrosine.
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