Structures of the CCR5 N Terminus and of a Tyrosine-Sulfated Antibody with HIV-1 gp120 and CD4

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The CCR5 co-receptor binds to the HIV-1 gp120 envelope glycoprotein and facilitates HIV-1 entry into cells. Its N terminus is tyrosine-sulfated, as are many antibodies that react with the co-receptor binding site on gp120. We applied nuclear magnetic resonance and crystallographic techniques to analyze the structure of the CCR5 N terminus and that of the tyrosine-sulfated antibody 412d in complex with gp120 and CD4. The conformations of tyrosine-sulfated regions of CCR5 (α-helix) and 412d (extended-loop) are surprisingly different. Nonetheless, a critical sulfotyrosine on CCR5 and on 412d induces a conserved site on gp120, which recognizes sulfotyrosine engenders posttranslational mimicry by the immune system.

Entry of human immunodeficiency virus type 1 (HIV-1) into host cells requires its gp120 envelope glycoprotein to bind to two cell-surface receptors, CD4 and a co-receptor, either CCR5 or CXCR4 [reviewed in (1, 2)]. CCR5 and CXCR4 are members of a family of chemokine receptors that are G protein-coupled receptors (3) characterized by seven transmembrane helices, an extracellular N terminus, which is variable in length, and three extracellular loops (ECLs) (Fig. 1A). The structure of the co-receptor has not been determined, but some insight has come from the crystal structures of other family members (4).

Elements critical to interactions with HIV-1 are located in the co-receptor N terminus and around its second extracellular loop (ECL2) (5–8). The co-receptor N terminus interacts with a highly conserved 4-stranded bridging sheet in gp120, which assembles upon CD4 binding, whereas the ECL2 region of the co-receptor interacts with the tip of the immunodominant V3 loop in gp120. Considerable distance separates these two interactive regions, which suggests that they are independent (9–12).

The N-terminal interaction of co-receptor with HIV-1 requires an unusual posttranslational modification, O-sulfation of tyrosine (13). On CCR5, tyrosines at residues 3, 10, 14, and 15 may be O-sulfated, but sulfations at residues 10 and 14 are sufficient to facilitate interaction with HIV-1 (14). Interestingly, many CD4-induced antibodies that react with the bridging sheet region are also modified by O-sulfation (15). To define structurally the interaction of HIV-1 with the N terminus of CCR5 and to understand the molecular details of the mimicry of this interaction by CD4-induced antibodies, we used a combination of nuclear magnetic resonance (NMR) and x-ray crystallography to determine the structures of the N terminus of CCR5 and of a functionally sulfated antibody, 412d, in complex with HIV-1 gp120. Analysis of these structures, combined with molecular docking and saturation transfer difference NMR, identified a conserved site on gp120, which recognizes sulfotyrosine with high selectivity.

We used NMR techniques that exploit the transfer of information from bound to ligand-free states (16, 17) to analyze the interactions of a 14-residue peptide (CCR5-1–15), which consisted of residues 2 to 15 of CCR5 with sulfotyrosine (Tys) at positions 10 and 14 (Fig. 1) (18). We collected two-dimensional (2D) nuclear Overhauser enhancement spectroscopy (NOESY) spectra of solutions containing CCR5-2–15 either free or in the presence of gp120, CD4, or a gp120-CD4 complex (peptide:protein ratio of 40:1). Whereas spectra containing free CCR5-2–15 or CCR5-2–15 with either gp120 or CD4 contained few cross peaks, CCR5-2–15 in the presence of the gp120-CD4 complex gave rise to high-quality spectra containing numerous NOEs (Fig. 1B and fig. S1). Complete 1H, 13C, and 15N assignments of CCR5-2–15 (table S1) were made on the basis of standard 2D homonuclear and heteronuclear NMR experiments that measure scalar and dipolar couplings.

The NOESY data of CCR5-2–15 in the presence of gp120-CD4 (Fig. 1B) were sufficient for calculating a high quality ensemble of NMR structures (Fig. 1C). Structure calculations were carried out on the ordered region comprising residues 7 to 15. A total of 70 distance constraints (corresponding to 35 intraresidue and 35 interresidue NOEs), and 56 dihedral angle restraints were included in the final round of structure calculations, which gave rise to an ensemble of 40 structures with a backbone root-mean-square deviation (rmsd) of 0.46 Å and an rmsd of 1.39 Å for all atoms in the ordered region (residues 9 to 14) (table S2). Superpositions of the final ensemble defined a helical conformation for residues 9 to 15, which deviated from the ideal by a backbone rmsd of only 0.26 Å (Fig. 1D). Sulfotyrosines 10 and 14 extended from the same face of the helix, with sulfate moieties separated by ~10 Å and an ~90° rotation around the helix axis.

We were unable to obtain crystals of CCR5-2–15 in complex with HIV-1 gp120-CD4, and the size and glycosylation of the ternary complex hindered direct determination by NMR. We were, however, able to obtain ~3.5 Å diffraction from crystals of the antigen-binding fragment (Fab) of the 412d antibody, in complex with gp120 (core with V3, CCR5-dependent isolate YU2) and CD4. The 412d antibody is functionally tyrosine-sulfated, binds to a CD4-induced epitope that overlaps the site of co-receptor binding on HIV-1 gp120, and recognizes preferentially CCR5-dependent strains of HIV-1 gp120 (15). Moreover, the tyrosine-sulfated region of 412d can be sub-
stituted for the tyrosine-sulfated region of CCR5 to create a chimeric 412d/CCR5 receptor that supports HIV-1 entry (19).

We solved the 412d-gp120-CD4 structure by molecular replacement. Despite less than optimal resolution and completeness, initial unbiased maps showed clear definition of important antibody features (fig. S2). Structure refinement resulted in an R cryst of 20% (Rfree 27%) (Fig. 2, table S3, and fig. S3). The overall mode of binding of 412d resembles that of 17b, which shares a heavy chain of similar genomic origin (fig. S4) (20). A hydrophobic interaction pins the second complementarity-determining region of the heavy chain (CDR H2) to a conserved hydrophobic surface on the bridging sheet of gp120, whereas the acidic CDR H3 binds a basic gp120 surface. Antibody 412d, however, interacts with a much larger overall surface area than either 17b or X5 (fig. S4). The increased 412d interaction surface is due primarily to an increase in buried surface associated with its CDR H3. Comparison of free (20) and bound structures of 412d shows that extensive ordering occurs in CDR H3 when bound to gp120 (fig. S5).

The two sulfotyrosines in the CDR H3 region of 412d bind to gp120 in quite different ways (Fig. 2). The sulfotyrosine at residue 100 of 412d (Tys 100412d) [Kabat numbering (21)] is mostly exposed, with its aromatic ring making p-cation interactions with the guanidinium of Arg 327gp120 and its sulfate group making only peripheral electrostatic interactions. By contrast, the side-chain of Tys 100c412d is mostly buried, with Ile 322gp120 and Ile 326gp120 embracing one face of the tyrosine ring, while the aliphatic base of Arg 440gp120 supports the other. Together, the two sulfotyrosines account for about 20% of the total buried surface on 412d, with almost 100 Å² derived from Tys 100c412d.

To facilitate interactions with the sulfotyrosines in 412d, the V3 stem is rearranged. The conserved Arg 298gp120 and Pro 299gp120 at the base of the V3 loop are mostly unchanged, but the subsequent Asn residues at 301gp120 and 302gp120 shift ~7 Å to form one wall of the Tys 100c412d sulfate-binding pocket. Residue 301gp120 is N-glycosylated, but the glycan faces solvent, and its presence should have little impact on the ability of the binding pocket to form. Meanwhile, in the returning strand (22), Ile

![Fig. 1. Structure of the tyrosine-sulfated N terminus of CCR5 in the gp120-bound conformation. (A) CCR5 sequence and schematic of its insertion in the cell membrane. Sequence letters in purple correspond to residues in CCR52–15, with sulfotyrosines (Tys) critical for interaction with HIV-1 highlighted in black. ECLs are labeled, and disulfide bridges (-SS-) depicted. (B) 2D NOESY spectra for CCR52–15 free in solution (left) and in the presence of gp120–CD4 (right). NMR samples (20 mM phosphate, 50 mM NaCl, pH 6.85) contained 800 μM CCR52–15 in the presence of 20 μM gp120–CD4 and were recorded at 500 MHz, 300 K, mixing time = 150 msec. Sequential NH(i)–CoH(i) NOEs were observed between every residue, thereby confirming sequential assignments, and predicted intraresidue NOEs were observed for all residues. No correlations beyond sequential NOEs were observed between residues 2 and 7, indicating that this region of CCR5 was extended or disordered. In contrast, NOEs from CoH(i) to NH(i + 1,2,3) and from NH(i) to NH(i + 1,2,3) were observed for residues 9 to 15 (fig. S1), indicating an ordered α-helical structure (33). (C) Structure of the ordered region of gp120-bound CCR52–15. Stereoview (left) of 25 lowest energy-simulated annealing structures superimposed by fitting to the backbone of residues 9 to 15. Structural statistics are provided in table S2. Backbone appears in blue, amide hydrogens (9 to 15) in blue, side chains (11 to 13) in green, and Tys 10 and Tys 14 in red. Ribbon diagram (right) of restrained minimized mean structure with side chains in stick representations.](www.sciencemag.org)
**Fig. 2.** Structure of the tyrosine-sulfated antibody 412d in complex with HIV-1 gp120 and CD4. (A) Ribbon representation. CD4 is yellow, the heavy chain of Fab 412d is dark blue, the light chain is cyan, and gp120 is gray, except for the V3 loop, which is orange. The CDR H3 loop of 412d is red, with sulftotyrosines depicted in stick representation. (B) Close-up, with molecular surface of gp120 in gray and sulftotyrosines of 412d (red labels) and select residues of gp120 (black labels) in stick representation. Dotted lines represent coordinating hydrogen bonds between gp120 and the sulfate group of Tys100c412d. The sulfate of Tys 100c412d makes a full complement of ionic interactions: a salt bridge to Arg 298gp120 and hydrogen bonds to the side-chain nitrogen of Asn 302gp120, the side-chain hydroxyl of Thr 303gp120, and the main-chain amides of 302gp120, 303gp120, and 441gp120 (34).

**Fig. 3.** Interaction of the N terminus of CCR5 with HIV-1 gp120-CD4. (A) Molecular docking. The 20 lowest energy structures (black) from 200 docking runs of CCR52-15 are shown in stick representation. Despite initial random orientations, all favorable docking solutions had Tys 14 binding at the bridging sheet-V3 interface; none had Tys 10 at this cleft. Ribbon representations illustrate CD4 in yellow, gp120 in gray (with V3 in orange), and the lowest energy structure of CCR57-15 in purple. (B) Close-up, with molecular surface of gp120 in gray and select residues of gp120 (black labels) and CCR5 (purple labels) in stick representation. (C) Saturation transfer difference NMR spectrum of CCR52-15 in the presence of gp120–CD4 (red) overlaid on a control 1H spectrum (black). Experimental conditions were identical to those used for NOE experiments, except that the carrier was set at -1 and 50 parts per million for on- and off-resonance saturation, respectively. The intensities of the most strongly enhanced peaks (Tys 14 and Tyr 15) have been normalized to the corresponding signals in the control spectrum. Peak assignments made by 2D NMR (table S1) appear above their corresponding doublet signals. Tys 14 and Tyr 15 show strong saturation transfer difference effects, whereas Tys 10 shows a medium effect and Tyr 3 a very weak effect. These effects correlate directly with the buried surface area of each tyrosine ring in the docked structure. See fig. S9 for overlaid spectra employing 1 to 7 s saturation. (D) Effect of CCR55-15 on the proteolytic sensitivity of the V3. Electrophoresis on an 8 to 25% gradient SDS polyacrylamide gel shows the results of thrombin digestion on gp120 (core with V3; YU2 R5 strain of HIV-1) alone, or in the presence of sCD4 or sCD4 and CCR52-15 (35). (E) Structural intermediates of HIV-1 entry. At far left, a single monomer of unliganded gp120 (gray) is shown with separated β-hairpins. The threefold axis, from which gp41 interacts in the functional oligomer, is labeled with the number 3. In the CD4-bound state, the bridging sheet assembles, and the V3 (orange) is exposed and flexible. The next state involves either (upper pathway) the interaction of the CCR5-ECL2 region with the V3 tip or (lower pathway) the interaction of the CCR5 N terminus, which induces rigidification of the V3 stem. Engagement of CCR5 at both N terminus and ECL2 region triggers additional conformational changes leading to HIV-1 entry.
H3 loop (residues 97 to 100f) produced an orientation, and multiple runs of the excised CDR would recapitulate the gp120 crystal structure. The gp120 region, which closely resembled its location and contacts in the crystal structure, interacted with the V3 stem. The top 10% of the solutions (20 best solutions) produced an energetically favorable interaction (−16.04 kcal/mol), which closely resembled its location and contacts in the crystal structure (Cr rmsd between crystal and docked CDR H3 was 1.03 Å) (fig. S6). We next docked the NMR structure of the CCR5 N terminus to the crystal structure of gp120-CD4. Multiple runs produced a cluster of energetically favorable solutions (−17.60 kcal/mol for the optimal solution), which placed CCR55-14 at the bridging sheet-V3 interface (Fig. 3, A and B). The top 10% of the solutions (20 best solutions) had rmsds of 1.04 Å (Cr) and 2.24 Å (all atoms). To validate the docked CCR5-CD4 structure, we performed saturation transfer difference NMR (17) on CCR55-14 in the presence of gp120-CD4. Control and difference spectra are shown in Fig. 3C. Contact surfaces of Tys and Tyr residues of CCR5 in the docked orientation correlated well with saturation transfer difference enhancements (Fig. 3C). We also observed good correlation between interacting residues in the docked gp120-CCR5 interface and gp120 and CCR5 substitutions (9, 25–27) that affect gp120-CCR5 binding (fig. S7).

The N terminus of CCR5 approaches from the same face of gp120 as CD4 but binds to an orthogonal surface at the intersection of the bridging sheet and the V3 loop (Fig. 3). The first CCR5 residues (Ser 7 and Pro 8) are ordered in the NMR structure interact with the V3 stem. In the helix (residues 9 to 15), Tys 10 interacts with the gp120 core and forms a salt bridge with Arg 327gp120. Asp 11 forms an ionic interaction with Arg 440gp120, Tys 14 is completely sequestered in the crevice between V3 and the bridging sheet, and the aromatic ring of Tyr 15 packs against Ile 439gp120 on the bridging sheet.

The structural rearrangements required to form the Tys 14 binding pocket would be expected to rigidify the V3 stem. We tested V3-proteolytic susceptibility (Fig. 3D). CD4 enhances V3-proteolytic susceptibility to thrombin (28, 29), whereas the combination of CD4 and CCR55-14 reduced proteolytic susceptibility (Fig. 3D), consistent with CCR5-rigidification of V3.

Overall, the gp120 recognition surface for CCR55-14 is much more highly conserved for CCR5-dependent isolates compared with those that use CXCR4. Good electrostatic complementarity is found between the acidic CCR55-14 and gp120, where the negatively charged C-terminal helix dipole is oriented toward the basic bridging sheet (fig. S8). The docked structure provides an explanation for the observed lack of order at the N terminus of CCR55-14, where CCR5 appears to extend away from gp120. At the C terminus, Tyr 15 points toward the target cell membrane where, in five residues, a disulfide would normally be made between the N terminus (Cys 20) and the third extracellular loop (Cys 269).

Despite the highly divergent tyrosine-sulfated structures of 412d and CCR5, a single sulfotyrosine (residue 100c in 412d and residue 14 in CCR5) is recognized in a similar manner by gp120 (Fig. 4). We used mutagenesis to probe the degree of similarity in this recognition (fig. S10). The alteration of a single nitrogen in a contact residue (Asn302Asp) in the conserved binding pocket ablates recognition of both 412d and CCR5, whereas a similar substitution (Asn300Asp), just outside the binding pocket, had little effect (30). The observed convergence of recognition likely reflects the high selectivity of this site for sulfotyrosine (a 7 Å deep pocket, with hydrophobic walls and a cationic floor, which is unlikely to interact favorably with other nonmodified amino acids). Such selectivity and favorable energetics bode well for design of therapeutics targeted at this site, because the gp120 residues that line the sulfotyrosine binding pocket are highly conserved for co-receptor binding.

The structure of the CCR5 N terminus with gp120-CD4 provides a further snapshot of the HIV-1 entry pathway (Fig. 3E). Before binding CD4, the bridging sheet is not formed and the V3 loop is occluded. Binding of CD4 induces bridging sheet assembly and V3 exposure. At this stage, the V3 is flexible and poised close to the target cell membrane. Subsequent interactions with CCR5 are still being elucidated. We show structural details for one: engagement by gp120 of the CCR5 N terminus, which requires formation of a conserved pocket for sulfotyrosine binding and converts the flexible V3 stem into a rigid β-hairpin. It will be interesting to integrate the order and timing of the rearrangements revealed here into the HIV-1 entry mechanism.
The Slit Receptor EVA-1 Coactivates a SAX-3/Robo–Mediated Guidance Signal in C. elegans

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The SAX-3/roundabout (Robo) receptor has Shiga-like toxin 1 (SLT-1)/Slit–dependent and –independent functions in guiding cell and axon migrations. We identified enhancer of ventral-axon guidance defects of unc-40 mutants (EVA-1) as a Caenorhabditis elegans transmembrane receptor for SLT-1. EVA-1 has two predicted galactose-binding ectodomains, acts cell-autonomously for SLT-1/Slit–dependent axon migration functions of SAX-3/Robo, binds to SLT-1 and SAX-3, colocalizes with SAX-3 on cells, and provides cell specificity to the activation of SAX-3 signaling by SLT-1. Double mutants of evo-1 or slit-1 with sax-3 mutations suggest that SAX-3 can (when slit-1 or evo-1 function is reduced) inhibit a parallel-acting guidance mechanism, which involves UNC-40/deleted in colorectal cancer.

The UNC-6/netrin guidance cue and its neuronal receptors, UNC-5 and UNC-40/deleted in colorectal cancer (DCC), are used in different combinations to guide growing axons toward (by attraction) or away (by repulsion) from the ventral nerve cord (VNC) of Caenorhabditis elegans (1). The incomplete penetrance of pioneer-axon guidance defects observed in unc-6/netrin and unc-40 single- and double-null mutants (Table 1) suggests that other mechanisms act in parallel with netrin signaling to guide axons toward the VNC. One such mechanism involves the Shiga-like toxin 1 (SLT-1)/Slit guidance cue, a large secreted protein with several predicted N- and O-glycosylation sites (2), and its receptor SAX-3, a homolog of the transmembrane (TM) roundabout (Robo) receptor (3–6). Both Drosophila and vertebrate Slit bind to Robo receptors (3, 7). C. elegans SLT-1/Slit is expressed predominantly by dorsal body-wall muscles and repels SAX-3/Robo–expressing AVM and PVM pioneer axons toward the VNC (2), concomitant with UNC–40–mediated attraction of these same axons toward the VNC by ventral sources of UNC-6 (1).

In C. elegans, slit-1 and sax-3 mutations affect the guidance of several of the same pioneer axons (8). For example, the pioneer axon of the lateral AVM sensory neuron in the anterior body extends toward and then along the VNC in wild-type (WT) animals (Fig. 1, A and B), but in slit-1 and sax-3 mutants, the AVM axon frequently grows directly toward the head (Fig. 1C). Cell-specific rescue experiments have demonstrated that sax-3(+)-dependent guidance of AVM axons is cell-autonomous (6, 8). Although SAX-3/Robo is the only previously known receptor for SLT-1, slit-1 mutants of C. elegans do not exhibit the nerve-ring and epithelial defects of sax-3/robo mutants, suggesting that SAX-3/Robo has both Slit-dependent and -independent functions in development (2).

We identified a TM protein, enhancer of ventral-axon guidance defects of unc-40 mutants (EVA-1), that is required to guide the AVM pioneer axon to the VNC (Fig. 1, A and B) by acting as a receptor for SLT-1. EVA-1 acts cell-autonomously, and ectopic expression of EVA-1 in SAX-3–expressing cells confers SLT-1 sensitivity to their migration. Thus, EVA-1 is predicted to be a receptor for SLT-1 that acts in conjunction with SAX-3 (as a likely co-receptor) to provide cell specificity for the activation of SAX-3 signaling by SLT-1. We also discovered a previously unknown in vivo function for SAX-3/Robo, which is to inhibit a signaling mechanism that normally functions in parallel to SLT-1 to guide pioneer axons along the dorsal/