Published in final edited form as:

J Mol Biol. 2011 July 29; 410(5): 778–797. doi:10.1016/j.jmb.2011.04.023.

The conformation and orientation of a 27-residue CCR5-peptide in a ternary complex with HIV-1 gp120 and a CD4-mimic peptide†

Einat Schnur^a, Eran Noah^a, Inbal Ayzenshtat^{a,1}, Hasmik Sargsyan^b, Tatsuya Inui^b, Fa-Xiang Ding^b, Boris Arshava^b, Yael Sagi^{a,2}, Naama Kessler^a, Rina Levy^a, Tali Scherf^c, Fred Naider^b, and Jacob Anglister^{a,*}

^aDepartment of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel

^bDepartment of Chemistry and Macromolecular Assembly Institute, College of Staten Island of the City University of New York, Staten Island, New York 10314, USA.

^cDepartment of Chemical Research Support, Weizmann Institute of Science, Rehovot 76100, Israel

Abstract

Interaction of CCR5 with the HIV-1 gp120-CD4 complex involves its amino-terminal domain (Nt-CCR5) and requires sulfation of 2-4 tyrosine residues in Nt-CCR5. The conformation of a 27-residue Nt-CCR5 peptide, sulfated at Y10 and Y14, was studied in both its free form and in a ternary complex with deglycosylated-gp120 and a CD4-mimic peptide. NMR experiments revealed a helical conformation at the center of Nt-CCR5(1-27) which is induced upon gp120 binding, as well as a helical propensity for the free peptide. A well-defined structure for the bound peptide was determined for residues 7-23, increasing by two-fold the length of Nt-CCR5's known structure. Two-dimensional saturation transfer experiments and measurement of relaxation-times highlighted Nt-CCR5 residues Y3, V5, P8-T16, E18, I23 and possibly D2 as the main binding determinant. A calculated docking model for Nt-CCR5(1-27) suggests that residues 2-22 of Nt-CCR5 interact with the bases of V3 and C4 while the C-terminal segment of Nt-CCR5(1-27) points towards the target cell membrane reflecting an Nt-CCR5 orientation that differs by 180° from a previous model. A gp120 site that could accommodate ^{CCR5}Y3 in a sulfated form has been identified. The present model attributes a structural basis for binding interactions to all gp120 residues previously implicated in Nt-CCR5 binding. Moreover, the strong interaction of sulfated ^{CCR5}Tyr14 with gp120 Arg440 revealed by the model and the previously found correlation between E322 and R440 mutations shed light on the role of these residues in HIV-1 phenotype conversion furthering our understanding of CCR5 recognition by HIV-1.

†This study was supported by NIH grant GM53329 to JA, a Pasteur-Weizmann grant to JA and BSF grant 2003220 to JA and FN. JA is the Dr. Joseph and Ruth Owades Professor of Chemistry and FN is the Leonard and Esther Kurtz Term Professor at the College of Staten Island.

© 2011 Elsevier Ltd. All rights reserved.

*To whom correspondence should be addressed. Jacob.Anglister@weizmann.ac.il. Phone: 972-8-9343394, Fax: 972-8-9344136.

¹Present address: Gonda Multidisciplinary Brain Research Center and Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel

²Present address: Stanford University, School of Medicine, Division of Oncology, Stanford, CA 94305, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCESSION NUMBERS

The structures and NMR data of Nt-CCR5(1-27) were deposited under PDB ID 2L87 and BMRB ID 17395.

Keywords

Sulfotyrosine; chemokine; NMR; protein structure; interactions

INTRODUCTION

Binding of the human immunodeficiency virus type 1 (HIV-1) to its target cells is mediated by the envelope glycoprotein gp120, located on the outer surface of the viral membrane¹. gp120 binding to CD4, a molecule found on the surface of both T-cells and macrophages, induces a conformational change in this envelope protein that exposes a chemokine receptor binding site^{2;3}. Association of gp120 with the chemokine receptor, usually CCR5 or CXCR4, promotes a global rearrangement of the trans-membrane envelope protein gp41 resulting in the fusion of the viral and cellular membranes and the entry of the viral capsid into the target cell^{4;5}. CCR5 and CXCR4 belong to the seven-transmembrane G protein-coupled chemokine receptor family⁴. CCR5, which binds macrophage inflammatory proteins 1 α and 1 β (MIP-1 α and MIP-1 β) and RANTES, is used by HIV-1 viruses designated R5 viruses, while CXCR4, which binds the stromal cell-derived factor-1 (SDF-1) as sole ligand, is used by HIV-1 strains designated X4 viruses.

Binding of gp120 to CCR5 involves the third variable region (V3) as well as the fourth constant region (C4) of the HIV-1 envelope glycoprotein gp120^{2;3;4;6;7}. HIV-1–neutralizing antibodies that bind the gp120 V3 loop or C4 region are able to block the binding of gp120-sCD4 (soluble CD4) complexes to CCR5-expressing cells and prevent the fusion of the virus with its target cells^{2;3}. The sequence of the V3 loop determines which specific chemokine receptors are used by different HIV-1 viruses³. Despite its name, the V3 region has conserved sequence elements and a conserved β -hairpin conformation, which the virus uses for co-receptor binding^{8;9;10;11}. Cormier and Dragic proposed a two-site binding mechanism of gp120 to the CCR5 co-receptor; the stem of V3 together with the C4 region mediate binding to the N-terminus of CCR5 (Nt-CCR5) while the V3 crown interacts with other extra-cellular loops of CCR5, most likely, the second extra-cellular loop¹².

The CCR5 chemokine receptor is expressed on subpopulations of lymphocytes, monocytes, macrophages and lymphoid organs¹³, and is also expressed in the central nervous system where it is found on neurons, astrocytes and microglia¹³. CCR5 has been implicated in diseases of the brain such as multiple sclerosis and Alzheimer's disease¹³. Mice and humans deficient in CCR5 do not exhibit detectable defects¹³ suggesting that other chemokine receptors can compensate for CCR5 and likely recognize its activators. CCR5 is of special importance in HIV-1 research^{14;15}, because it binds R5 strains, which are responsible for HIV-1 transmission in most cases and are the predominant virus phenotype until the symptomatic stage of the disease¹⁵. Individuals with a 32-base-pair deletion in the CCR5 gene that leads to expression of a non-functional receptor, appear to be highly resistant to HIV-1 infection^{16;17;18}. This observation suggests that inhibitors targeting the interaction of gp120 with CCR5 should have excellent potential as therapeutic agents against AIDS (Acquired immune deficiency syndrome)¹⁹. An example of such an inhibitor is the CCR5 antagonist Maraviroc, a recently approved drug for the treatment of AIDS and HIV-1 infection²⁰.

Site-directed mutagenesis as well as biochemical and immunological techniques have identified the N-terminus (Nt-CCR5) and the second extra-cellular loop (ECL2) of CCR5 as most important for binding to CC-chemokines and HIV-1 gp120^{21;22;23;24}. The major binding determinant is within a region rich in tyrosine and acidic amino acids in Nt-CCR5^{22;25}. An unusual posttranslational modification, O-sulfation of two or more of the

tyrosine residues in Nt-CCR5 is essential for optimal chemokine receptor and HIV-1 co-receptor function of CCR5^{26; 27; 28}.

Peptides corresponding to residues D2-E18 or the first 22 residues of CCR5 caused 50% inhibition of gp120/CD4 binding to CCR5 expressing cells at 100 μ M and 50 μ M concentration, respectively, provided they were sulfated at Y10 and Y14^{26; 28}. Both unsulfated and tyrosine phosphate homologues were ineffective²⁸. The dissociation constant for the ternary complex of the entire CCR5 with a gp120/CD4 complex was 5 nM²⁹, approximately three to four orders of magnitude smaller than measured for the disulfated 22-residues Nt-CCR5 peptide^{26; 30}. Thus, while Nt-CCR5 contributes considerably to the binding energy, the data indicate that other determinants of CCR5 must also be involved.

Understanding the interactions between gp120 and its receptors at atomic resolution would be facilitated by the determination of either an X-ray or an NMR structure of their complexes. However, with current technology such studies are very difficult because the receptors are integral membrane proteins. Recently, the structure of the chemokine receptor CXCR4 was solved³¹. However, the structure of its N-terminus (residues 1-26), which plays a major role in HIV-1 binding, did not have interpretable density and thus could not be determined. Therefore, alternative approaches are necessary. Studies discussed above established the importance of the N-terminal region of CCR5 in the interaction between HIV-1 and macrophages, and the fact that synthetic peptides can selectively compete with this interaction. Based on these conclusions Nt-CCR5 peptides can and have been used as surrogates for CCR5 in structural studies.

The crystal structure of gp120 in complex with CD4 and 412d Fab which contains sulfated tyrosine residues was solved by x-ray crystallography and NMR methods were used to determine the bound conformation of a 14-residue Nt-CCR5(2-15) peptide with sulfotyrosine (Tys) at positions 10 and 14³². Only nine residues (⁷SPIYDINYY¹⁵) of this peptide were found to be structured and residues ⁹YDINY¹⁴ formed a short helix. Since residues D2, D11, S17, E18 and C20 were found by site-directed mutagenesis to be important for CCR5-mediated viral fusion and entry^{28; 33} and there are indications that residues in the segment Q21-K26 also contribute to gp120 binding^{22; 33}, we studied the conformation and interactions with gp120 of a considerably longer Nt-CCR5 peptide comprising 27 residues, Nt-CCR5(1-27), sulfated at Y10 and Y14.

To facilitate future NMR structural studies of the ternary complex of gp120/CD4/Nt-CCR5(1-27) and elucidate the interactions between gp120 and CCR5, the size of the complex must be as small as possible. Unfortunately, even the most favorable gp120 molecule (after removal of the V1/V2 loops and 90% of the glycosidic appendages, ~45 kDa), results in a 70 kDa complex after binding to sCD4 (a 21 kDa two-domain soluble entity) and an Nt-CCR5(1-27) peptide (~3 kDa). However, Vita and co-workers designed a 27-residue mini-protein designated CD4M33 (hereafter referred to as ^{mi}CD4), which successfully mimics the biological function of sCD4³⁴. Its affinity to gp120 (4 nM) is of similar order of magnitude as that of sCD4 to gp120, and the structures of gp120 in complex with the ^{mi}CD4 peptide and sCD4 are almost identical, exhibiting an RMSD of 0.89 Å³⁵. Most importantly, this mini-protein induces conformational changes in gp120, and enhances binding of gp120 to CCR5 similarly to sCD4. Thus, ^{mi}CD4 offers an attractive lower-molecular weight alternative to sCD4 in structural studies of ternary gp120 complexes by NMR spectroscopy. It is important to emphasize that although ^{mi}CD4 and Nt-CCR5(1-27) are peptides, they manifest the biological activities of their respective intact proteins, and therefore structural studies on the ternary complex of ^{mi}CD4, Nt-CCR5(1-27) and gp120 yield physiologically relevant information.

Our results reveal a well-defined structure for residues 7-23 of Nt-CCR5(1-27) bound to gp120^{mi}CD4 which is made up of two helical elements, the first spans residues 9-17 and the second is made of residues 20-23. This structure increases by almost two-fold the length of the previously reported structure of Nt-CCR5(2-15)³². Moreover, the propensity of Nt-CCR5(1-27) to adopt a helical conformation was found also in its free form. Finally, we identified the segment Y3, V5, P8-T16, E18, I23 and possibly D2 of Nt-CCR5(1-27) as the major binding determinant to the gp120^{mi}CD4 complex. A calculated docking model for Nt-CCR5(1-27) revealed an extensive surface formed by the gp120 C4 and V3 regions that interacts with residues D2-K22 of Nt-CCR5 while the C-terminal segment of Nt-CCR5(1-27) is flexible and continues towards the putative location of the target cell membrane. A new gp120 binding pocket that could accommodate CCR5^{Y3} in a sulfated form has been revealed. In our docking model the orientation of the N-terminal segment of CCR5 differs by 180° from that in a previous model proposed by Kwong, Bewley and co-workers³². Unlike the previous model the present one attributes a role in binding interactions to all gp120 residues implicated in Nt-CCR5 binding by biochemical studies. In addition the strong electrostatic interaction between gp120^{R440} and CCR5^{Y^S14} and the previously found correlation between a negatively charged residue at gp120 position 322 and a positively charged residue at position 440 explains how E322 and R440 determine CCR5-tropism.

RESULTS

Conformation of Free Nt-CCR5(1-27)

Before initiating studies of the structure of Nt-CCR5(1-27) in complex with gp120 we characterized the conformation of the free peptide. This was done because we wanted to compare and contrast the structure of the free and complexed peptide. When NOESY measurements were carried out with Nt-CCR5(1-27) at 300 K without NaCl added, only a few medium-range NOE cross peaks were observed (Figure 1a). However, when NOESY measurements were carried out with Nt-CCR5(1-27) at 274 K (Figure 1b), several NOE interactions characteristic of a helical structure were detected, some of which are explicitly labeled in Figure 1. These NOEs include several H_N(i+3)-H_α(i) NOE interactions: D11(H_N)-P8(H_α), Y15(H_N)-I12(H_α), T16(H_N)-N13(H_α) and A20(H_N)-S17(H_α) (Figure 2b). H_N(i+2)-H_α(i) -type NOEs were observed between residues V5-Y3, S7-V5, I9-S7, D11-I9, Y^S14-I12, T16-Y^S14, S17-Y15, E18-T16, A20-E18, N24-K22 and K26-N24 (Figure 2b). Thus at low temperature Nt-CCR5(1-27) exhibits medium-range backbone connectivities indicative of a helical propensity for many of the peptide's residues. This tendency to form a helical structure extends beyond residues I9-Y15 and includes also residues T16-E18 and A20-I23.

Conversely, the deviations of H_α, C_α and C_β chemical shifts from random coil values for free Nt-CCR5(1-27) (the carbon shifts were measured at ¹³C natural abundance; see Materials and Methods) did not show any significant helical tendency indicating that the observed medium-range NOEs originate from a small population of helical peptide or that the peptide adopts multiple turn conformations rather than a helical structure.

Despite the NMR evidence for a helical propensity of the middle residues (9-20), the circular dichroism (CD) spectrum of Nt-CCR5(1-27) measured to assess its secondary structure at 274 K indicated a predominantly disordered structure (Data not shown). It should be pointed out that the CD technique measures the average environment for all of the peptide chromophores in Nt-CCR5(1-27) and that little information is available on the contribution of Tyr(SO₃) residues to the far UV CD.

Characterization of gp120

Two gp120 variants lacking the gp41 binding segments as well as the V1 and V2 variable loops (⁸⁸⁻⁴⁹²gp120ΔV1/V2(N301Q;T388A)) were prepared in our laboratory; a 56 kDa gp120 construct that is homogeneously glycosylated with short carbohydrate chains, Man₅GlcNAc₂, (glycosylated-^{tr}gp120) and a 40 kDa gp120 in which the carbohydrate chains were cleaved with ENDO-H_f, leaving a single GlcNAc- at each glycosylation site (deglycosylated-^{tr}gp120). The oligomerization state of our deglycosylated-^{tr}gp120 construct was first evaluated using a series of FPLC runs conducted at different NaCl concentrations (50, 300, 500 mM) and different pH values (6.0, 7.0 and 8.0). At low salt concentration (50 mM), the major peak in the FPLC was associated with a high molecular-weight large aggregate, while the monomer/dimer associated peaks were relatively small. At 300 and 500 mM salt, the opposite was observed, i.e. the great majority of the protein eluted at the peak associated with the monomeric state (with a higher molecular weight shoulder). Deglycosylated-^{tr}gp120 also appeared to aggregate at pH 6.0, while reduced aggregation was observed at pH 7.0 or 8.0.

In order to further assess the oligomerization state of both our ^{tr}gp120 constructs, we also carried out sedimentation equilibrium measurements ³⁶. Both ^{tr}gp120 variants were complexed with ^{mi}CD4 and experiments were performed in the presence of different salt concentrations (0, 50 or 300 mM NaCl). The data was fitted to a single ideal species model. For both glycosylated- and deglycosylated-^{tr}gp120/^{mi}CD4 the fit indicated a molecular mass much higher than expected for a monomer. All fits exhibited systematic errors in the calculated residuals indicating aggregation of gp120. Comparison of the analysis for both forms of gp120 reveals that the monomeric form of ^{tr}gp120 is more favorable at higher salt concentrations. Deglycosylated-^{tr}gp120/^{mi}CD4 was more prone to aggregation without the addition of NaCl. Despite a lower molecular weight for its monomeric form, in the absence of salt, deglycosylated-^{tr}gp120/^{mi}CD4 gave rise to a higher molecular weight species than glycosylated-^{tr}gp120/^{mi}CD4 (193 kDa and 142 kDa, respectively). These results lead us to conclude that NMR measurements of deglycosylated-^{tr}gp120/^{mi}CD4 should be performed at pH 7 and at high salt concentration, since under these conditions the aggregation in our system is less severe.

Binding of Nt-CCR5(1-27) to ^{tr}gp120/^{mi}CD4

As mentioned above several residues in the CCR5 segment T16-K26 were previously implicated in gp120 binding ^{21; 33}. Therefore we wanted to characterize the conformation of Nt-CCR5(1-27) in complex with gp120 and identify the peptide residues interacting with the envelope protein. To examine the suitability of the ^{tr}gp120/^{mi}CD4 complex with the Nt-CCR5(1-27) peptide for structural studies, we initially characterized ^{tr}gp120 binding to the ^{mi}CD4 peptide by surface plasmon resonance (SPR). Binding experiments were conducted on both glycosylated-^{tr}gp120 and deglycosylated-^{tr}gp120. For the SPR studies a biotin was linked to the ^{mi}CD4 peptide as described previously ³⁴, and binding was tested at 150 and at 300 mM NaCl. At 150 mM NaCl K_D values of 12 and 28 nM were calculated for glycosylated-^{tr}gp120 and deglycosylated-^{tr}gp120, respectively. Similar values, 14 and 20 nM, were calculated at 300 mM NaCl, respectively. These results are in good agreement with previously published results ³⁴ and comparable to equilibrium dissociation constants obtained with soluble CD4 (K_D = 3 or 22 nM) ^{37; 38}.

Subsequently the binding of the ^{tr}gp120/^{mi}CD4 complex to Nt-CCR5(1-27) was tested. Biotin was linked to the side chain of a lysine residue added to the peptide C-terminus through a three residue flexible linker, Gly-Ser-Gly (See Materials and Methods) to enable binding to a streptavidin containing matrix. The biotin was linked to the C-terminus of the peptide rather than to the N-terminus. This mimics the native topology of the N-terminus in

the context of the entire CCR5 in which the N-terminus is far from the membrane spanning region and the C-terminus of the N-terminal segment is followed by the membrane spanning region. The binding to Nt-CCR5(1-27) was tested for the two ^{125}I -gp120 proteins and for their complexes with ^{125}I -CD4 in 50 mM and 300 mM NaCl. At 50 mM NaCl K_D values of $1.4 \pm 0.3 \mu\text{M}$ and $0.7 \pm 0.2 \mu\text{M}$ were determined for glycosylated- and deglycosylated- ^{125}I -gp120/ ^{125}I -CD4, respectively, which is in good agreement with literature results on similar peptides^{26; 30}. The two fold difference in binding affinity between the glycosylated and deglycosylated forms of ^{125}I -gp120 may reflect some shielding effects of the carbohydrate moieties. At 300 mM NaCl, an increase of one order of magnitude in K_D (weaker binding) was observed for deglycosylated- ^{125}I -gp120/ ^{125}I -CD4 while no binding could be detected for glycosylated- ^{125}I -gp120/ ^{125}I -CD4. Binding of glycosylated- ^{125}I -gp120 (without ^{125}I -CD4) to immobilized Nt-CCR5(1-27) was negligible at both salt concentrations while binding of deglycosylated- ^{125}I -gp120 (without ^{125}I -CD4) to immobilized Nt-CCR5(1-27) was negligible at 300 mM NaCl, but gave rise to a measurable signal for deglycosylated- ^{125}I -gp120 concentrations between 5 and 0.5 μM . Weaker binding as a result of increased salt concentration is expected in systems where electrostatic interactions are the driving force for binding of the components, as in our gp120 and sulfated-Nt-CCR5(1-27) system. Fortunately, NMR is well suited to explore weak binding. Moreover, the NMR methods applied in this study depend on fast off-rates to transfer binding information from large, high molecular weight complexes that are hard to detect by NMR, to easily detectable small moieties.

Secondary Structure of Nt-CCR5(1-27) Bound to Deglycosylated- ^{125}I -gp120/ ^{125}I -CD4

In a previous study, Bewley and co-workers determined the structure of the fourteen-residue-Nt-CCR5(2-15) peptide bound to gp120 with native long and non-homogenous carbohydrate side-chains using transferred-NOE measurements carried out at 300 K, pH 6.85 in the presence of 50 mM NaCl³². For studying the conformation and interactions of Nt-CCR5(1-27) we chose to carry out measurements using the deglycosylated- ^{125}I -gp120/ ^{125}I -CD4 complex in the presence of a 10-fold excess of the longer peptide Nt-CCR5(1-27) and 300 mM NaCl. Measurements at different temperatures were found useful to resolve amide-proton cross-peak overlaps in the NOESY spectra. For example, the chemical shift values of the amide protons of D11 and Y3 overlap at 300 K but at 274 K, they differ by 55 Hz. Figure 3a presents the $T_{1\rho}$ -filtered NOESY spectrum of Nt-CCR5(1-27) bound to deglycosylated- ^{125}I -gp120/ ^{125}I -CD4 at 300 K. In this experiment only signals from the small peptide appear since the broad proton resonances of the deglycosylated- ^{125}I -gp120/ ^{125}I -CD4 vanish due to the short relaxation times of the protons of hetero-dimeric complex and the use of an appropriate $T_{1\rho}$ -filter. Spectra of the peptide in the presence of the deglycosylated- ^{125}I -gp120/ ^{125}I -CD4 complex and of the free peptide under identical conditions are shown in Figure 3a and 3b, respectively. The bound Nt-CCR5(1-27) gave rise to high-quality spectra containing many strong NOEs, while Nt-CCR5(1-27) in its free state at 300 mM NaCl gave rise to a spectrum with only inter-molecular and sequential NOEs at 300 K. Several control experiments were carried out to verify that the interactions we observe in the $T_{1\rho}$ -filtered NOESY spectrum indeed originate mostly from the bound conformation. $T_{1\rho}$ -filtered NOESY experiments were collected on Nt-CCR5(1-27) in the presence of glycosylated- ^{125}I -gp120/ ^{125}I -CD4 and on Nt-CCR5(1-27) in the presence of deglycosylated- ^{125}I -gp120 without ^{125}I -CD4. Both control experiments were carried out under the same conditions as for Nt-CCR5(1-27) in the presence of deglycosylated- ^{125}I -gp120/ ^{125}I -CD4 (As mentioned above no binding of Nt-CCR5(1-27) to the two control complexes could be detected by SPR). In both control experiments, only sequential cross peaks were observed. Thus, all medium-range interactions we observe in the $T_{1\rho}$ -filtered NOESY spectrum of Nt-CCR5(1-27) in the presence of deglycosylated- ^{125}I -gp120/ ^{125}I -CD4 complex at 300 K and 300 mM NaCl are transferred-NOE cross-peaks that originate from the bound conformation and

are therefore not seen in the NOESY spectrum of the free peptide under the same conditions nor in the Nt-CCR5(1-27) peptide in the presence of proteins that exhibit no detectable binding to the peptide.

Numerous medium-range NOE interactions, typical of a helical conformation, were identified for Nt-CCR5(1-27) bound to deglycosylated-^{tr}gp120/^{mi}CD4. Among these are H_N(i+3)-H_α(i) NOEs between residues Y^S10-S7, D11-P8, I12-I9, N13-Y^S10, Y15-I12, T16-N13, A20-S17, K22-P19, I23-A20 and K26-I23, as well as H_N(i+2)-H_N(i) interactions between Y^S14-I12 and between T16-Y^S14 and, most importantly, an H_N(i+4)-H_α(i) NOE interaction between S17-N13. Some of these crosspeaks are explicitly assigned in Figure 3a while all are summarized in Figure 2a. Upon binding to deglycosylated-^{tr}gp120/^{mi}CD4, the tendency of Nt-CCR5(1-27) to fold into a helix is increased considerably. The peptide structure is stabilized as evidenced by the increased number of connectivities seen in the spectrum of the ternary complex compared to those seen in the unbound peptide even at low temperature with no added NaCl. Overall there are 27 medium-range NOE interactions for the free peptide at 274 K and 46 interactions characteristic of a helical conformation in the presence of deglycosylated-^{tr}gp120/^{mi}CD4 at 300 K. Moreover, the αN(i,i+4) interaction between residues 17^{HN} and 13^{Hα} and the NN(i,i+2) interactions between residues 14-12 and 16-14 are present only in the spectrum of the bound peptide (Figure 2).

Structure of Nt-CCR5(1-27) Bound to Deglycosylated-^{tr}gp120/^{mi}CD4

An ensemble of the 10 lowest-energy NMR-derived structures of the peptide bound to the deglycosylated-^{tr}gp120/^{mi}CD4 complex is shown in Figure 4. The structural statistics are presented in Table 1. Eighty six medium-range interactions were incorporated in the structure calculation, out of which 46 are typical of a helical conformation as described above. The backbone RMSD of the 10 lowest energy structures (out of 50 accepted structures) is 0.6 ± 0.3 Å for residues P8-S17 and 1.5 ± 0.6 Å for residues S7-I23.

The secondary structure tendencies of residues in the 10 lowest energy calculated structures were analyzed by MOLMOL³⁹ using H-bonding patterns⁴⁰. The segment ⁸PIYSDINYSYTS¹⁷ exhibits a helical conformation in which residues P8-N13 are found in a ₃₁₀-helix and residues Y^S14-S17 in an α-helical conformation. Another possible helical turn, comprising the segment ²⁰AQKI²³, was also identified. Despite its deviation from ideal helicity, the structure of this segment of the peptide is well-defined as judged by the RMSD. The tendency to form a helical structure includes also residues 24-26 as evidenced by the 26^{HN}-23^{Hα} interaction in the NOESY spectrum of the bound peptide (Figure 2). However, the structures for this segment did not converge.

A few peaks in the T_{1ρ}-filtered NOESY spectra could not be assigned unambiguously and therefore were not taken into account in structure calculations. Thus it is possible that the structure of Nt-CCR5(1-27) bound to gp120 has additional features that have not been resolved in our study.

Residues of Nt-CCR5(1-27) Interacting with Deglycosylated-^{tr}gp120/^{mi}CD4

Saturation transfer difference (STD) NMR relies on the transfer of saturation from the protein to the free ligand. Irradiation of a protein resonance at a frequency where no ligand resonance is present leads to very efficient saturation of the entire protein by spin diffusion. Saturation is also transferred to bound-ligand protons of residues interacting with the protein. The exchange between bound and free ligand results in observation of saturation transfer to the free ligand protons. The degree of saturation of an individual proton of the small ligand molecule reflects the proximity of this proton to the protein surface in the complex. That is, an STD experiment results in a difference spectrum where free-ligand

proton signal intensities are proportional to the proximity of the bound ligand-protons to the receptor^{41; 42}. 1D-STD and 2D-HOHAHA-STD spectra were collected on a sample containing a 1:10 ratio of deglycosylated-^{tr}gp120/^{mi}CD4 to Nt-CCR5(1-27), while irradiating at -2 ppm, and pronounced STD signals were indeed observed. Control experiments with the same irradiation scheme were carried out on samples containing both Nt-CCR5(1-27) in the presence of glycosylated-^{tr}gp120/^{mi}CD4 and also Nt-CCR5(1-27) alone, and no STD peaks were observed for both controls (data not shown). The strongest enhancements observed for Nt-CCR5(1-27) interacting with the deglycosylated-^{tr}gp120/^{mi}CD4 complex, were of aromatic protons of Tyr residues. Figure 5a presents the aromatic region of the 1D-STD spectrum, in which the most pronounced peaks are of the sulfated Tyr residues, Y10 and Y14 (>90%). Y15 is also strongly affected and Y3 to a lesser extent, although the saturation transfer to Y3 (50%) is significantly above the cutoff (25%).

The great disadvantage of a 1D-STD spectrum is the severe overlap especially for non-aromatic side chain protons (Figure 5b). In most cases it is impossible to unambiguously assign a peak in a 1D-STD spectrum to a single atom in a specific residue. Expanding the measurement into a second dimension makes it possible to unambiguously assign most of the STD signals. In the 2D-STD-HOHAHA spectrum, in which only peaks of Nt-CCR5(1-27) protons that are in close proximity to the deglycosylated-^{tr}gp120/^{mi}CD4 complex appear (Figure 6), every residue gives rise to multiple cross peaks. The strongest enhancement observed for each residue spin-system is presented in Figure 7 where the saturation transfer in both 1D-STD and 2D-STD data is presented. Unambiguous assignment of 1D-STD signals was aided by results from the 2D-STD-HOHAHA spectrum and is presented in Figure 7 as grey bars. In some case the much higher digital resolution of the 1D STD spectrum in the acquisition dimension in comparison with the 2D STD enabled us to resolve signal overlap in the 1D spectrum. Based on both the 1D and 2D-STD we conclude that residues Y3, V5, P8, I9, Y^S10, D11, I12, Y^S14, Y15, T16, I23, D2 and/or N13 and Q4 and/or E18 exhibits enhancements greater than 25%. The quantification of the STD enhancements in both the 1D and 2D-STD spectra highlights a segment of Nt-CCR5(1-27) consisting of residues Y3, V5, P8-T16 and I23 which is most affected by interaction with gp120. Also residues Q4 and/or E18 and D2 and/or N13 may possibly interact with gp120.

To further characterize the binding determinant of Nt-CCR5(1-27), we used a series of $T_{1\rho}$ -filtered HOHAHA experiments to determine the $T_{1\rho}$ relaxation time⁴³ of the free and bound Nt-CCR5 peptide. In principle, peptide protons strongly interacting with a large protein are immobilized upon binding and assume a $T_{1\rho}$ relaxation time comparable to that of the protons of the protein. Peptide protons with no interaction with the large protein retain some mobility and have considerably longer $T_{1\rho}$ relaxation time^{43; 44; 45}. To characterize the $T_{1\rho}$ relaxation time, $T_{1\rho}$ -filtered HOHAHA experiments with the same mixing time but with an additional spin-lock inserted after the first 90° pulse were measured with spin-lock pulse times varying between 0-200 ms in 40 ms increments. The $T_{1\rho}$ -filtered HOHAHA spectra were measured on both a sample containing Nt-CCR5(1-27) alone and on a sample in which substoichiometric amounts of Nt-CCR5(1-27) (70%) relative to deglycosylated-^{tr}gp120/^{mi}CD4 were used to minimize interference from free ligand protons. The decay in signal intensity with increasing spin-lock length was used to extract the $T_{1\rho}$ relaxation times for each Nt-CCR5(1-27) residue in the free and bound state (Figure 8). Most of the Nt-CCR5(1-27) C- terminal residues, A20, Q21, N24, V25, K26 and Q27 and also V5 and S6 exhibited longer $T_{1\rho}$ relaxation times, revealing residual flexibility, while residues Y3, I9, Y^S10, D11, N13, Y^S14, Y15 and T16 showed significant (> 40 %) decreases in $T_{1\rho}$ relaxation times, suggesting that they are part of the determinant recognized by gp120. S6 and E18 exhibited a slightly smaller decrease in relaxation time than the residues in the segment I9-T16 indicating that they are at the boundary of the core epitope

recognized by gp120. Interestingly D11 and Y15 exhibited slightly smaller decrease in relaxation time in comparison with adjacent residues in the core epitope I9-T16 suggesting that in comparison with the other epitope residues they have increased mobility. Y3 is unique at the N-terminal end of Nt-CCR5(1-27), exhibiting considerable shortening of $T_{1\rho}$ in comparison with S6. Taken together with the STD results for Y3, these two observations suggest that Y3 may be more highly immobilized than the other residues in this otherwise flexible segment of the gp120-bound Nt-CCR5(1-27) peptide.

Docking Model of gp120/Nt-CCR5(1-27)

In-silico docking of Nt-CCR5(1-27) to gp120 was performed using HADDOCK⁴⁶. Active residues of gp120 used in the HADDOCK procedure (residues that are known to make contact within the complex) were those found to be involved in CCR5 binding by mutagenesis and those inferred to be involved in such interactions according to the crystal structure of Fab 412d/gp120/sCD4 complex (residues R298, N301, N302, T303, R304, K305, I323, I324, G325, D326, I327, R328, R419, I420, K421, Q422, R440 and R444; numbering according to the HXB2 HIV-1 strain)^{12; 32; 47}. Active residues of Nt-CCR5(1-27) were those found to participate in binding by our STD-NMR and $T_{1\rho}$ measurements as well as previous biochemical studies^{21; 22; 33; 48}. The lowest energy structure of the complex is presented in Figure 9.

Examination of the docking model reveals that the stems of the V3 and the C4 loops create an elongated positively charged face consisting of R298, R328, R419, K421 and R440 (Figure 10a). Several isoleucine residues are almost evenly dispersed on the surface and include I323, I327, I423 and I424. As shown by the helical wheel presented in Figure 10b the Nt-CCR5 helical segment P8-S17 forms a complementary negatively charged surface made of Y^S10, D11 and Y^S14. In the model the sulfated-Tyr14 of Nt-CCR5(1-27) occupies the same binding pocket occupied by Tys 100c of the Fab 412d in complex with gp120³². This pocket is lined with two positively charged residues, gp¹²⁰Arg298 and gp¹²⁰Arg440, two isoleucines, gp¹²⁰Ile323 and gp¹²⁰Ile327 and polar residues, gp¹²⁰Asn302 and gp¹²⁰Thr303. Interestingly, our docking-model revealed a second potential binding pocket for sulfotyrosine, similar in amino acid composition to the tyrosine-sulfate pocket described before. This second pocket is made of the gp120 segment⁴¹⁹RIKQI⁴²³ on C4 and partially interacts with^{CCR5}Y3 (Figure 9). The specific interactions formed between gp120 and the CCR5 peptide involve 15 intermolecular hydrogen bonds and salt bridges between residues 8, 11, 14, 15, 18 and 22 of Nt-CCR5(1-27) and residues 298, 300, 302, 303, 305, 322, 323, 328, and 440 of gp120. Table 2 summarizes the statistics of the model calculated by HADDOCK and the interactions between the proteins.

Despite evidence from STD and $T_{1\rho}$ -filtered NMR experiments, which highlight the role for^{CCR5}Y^S10 in gp120 binding, the docking model reveals that, unlike Tys14 which shows numerous interactions with gp120, Y^S10 only interacts with gp¹²⁰Arg328. In this respect, Y^S10 is similar to Tys100 of the antibody 412d which in the crystal structure shows only peripheral electrostatic interactions with gp120 (see Discussion).

The C-terminal segment of Nt-CCR5(1-27) (residues 24-27) does not interact with gp120. Rather, it extends away from gp120 and continues towards the putative location of the target cell membrane. This orientation of Nt-CCR5 is enabled by the bend formed by P19 which also allows the electrostatic interaction between^{CCR5}K22 and gp¹²⁰E322.

DISCUSSION

Mapping of the Nt-CCR5(1-27) determinant recognized by gp120

In this study we mapped the Nt-CCR5(1-27) determinant recognized by gp120 to residues Y3, V5, P8-T16, E18 and I23 and possibly D2. This mapping has been carried out using 1D- and 2D-STD measurements^{41; 42} as well as T_{1ρ}-relaxation times measurements^{43; 44; 45}. Of the residues comprising the binding determinant, the sulfated Y10 and Y14 play a very important role as demonstrated by the strong saturation transfer to the aromatic rings of these residues. Interestingly Y3 was found to interact with gp120 and it interacts with a site similar in its amino acid composition to the pocket occupied by the sulfated tyrosine Y^S14 suggesting that Y3 in CCR5 could be sulfated.

Among the residues highlighted as being involved in binding, D2, Y3, Y10, D11, Y14, Y15, E18 were previously identified by alanine-scanning mutagenesis to be important for interactions with gp120^{21; 22; 33; 48}. Mutations of P8 and N13 were found to have a milder effect on HIV binding⁴⁸ and the role of Q21^{22; 33} and K22^{21; 22} of CCR5 in gp120 binding was equivocal. Previous studies indicated that Q4, V5, S6, S7, I9, I12 and T16 when mutated to alanine, did not impair co-receptor activity, and residues I23 and V25 were not studied by site-directed mutagenesis^{21; 22; 33; 48}. Thus our conclusions regarding the involvement of V5, I9, I12 and T16 in interactions with gp120 do not agree with the previous site-directed mutagenesis studies. It is possible that interactions of Y3, Y^S10, Y^S14 and Y15 with gp120 limit Nt-CCR5(1-27)'s mobility and result in saturation transfer to nearby residues such as V5, I9, I12 and T16. Another possibility is that mutation of valine, isoleucine and threonine to alanine does not cause significant change in binding because the sidechain of alanine does not represent a drastic change either in polarity of the side chains or its the size of the mutated residues in contrast to a change from lysine to alanine.

In contrast to the present study Y3 did not exhibit significant saturation transfer in previously reported NMR studies^{32; 49} that used the fourteen-residue peptide Nt-CCR5(2-15). This could be the result of removing one N-terminal residue in the previously studied peptide and the resulting proximity of D2 and Y3 to the positively charged N-terminus of Nt-CCR5(2-15), which might interfere with the interaction of positively charged residues in the gp120 C4 with D2 and Y3 in Nt-CCR5. The involvement of the side chains of non-aromatic amino acid residues of Nt-CCR5 in gp120/CD4 interactions was not revealed previously because these could not be assigned in the 1D-STD spectrum.

The structure of Nt-CCR5(1-27)

A well-defined structure for the segment S7-I23 was obtained in the present study on the basis of NMR distance and dihedral angle constraints. Two helices formed by the segments P8-S17 and A20-I23 were identified although the structure of the latter helical segment deviates from an ideal helical conformation. Deviation from an ideal helix in the segment A20-I23 (despite the overall convergence of structures exemplified by the low RMSD) could result from its shortness and insufficient number of NOE constraints due to spectral overlap.

Although the binding determinant includes residues Y3, V5 and possibly D2, a well-defined structure was observed only for residues S7-I23. This is because in the absence of long-range interactions only helical conformations and reverse-turns structures can be determined by NMR for short linear peptides. Single β-strands manifest no medium-range connectivities and are not revealed for a single peptide chain that does not assume a hairpin structure. Definition of the structure of the segment D2-S6 will require measurements of interactions between gp120 and Nt-CCR5(1-27).

We found that free Nt-CCR5(1-27) has a propensity to form a helical conformation although this helix is transient and, consistent with CD studies, only a small population of the conformational distribution of this peptide reflects this structure. The propensity to form a helical conformation can be detected at 300 K with no NaCl added and is increased at a lower temperature (274 K). The tendency of the free 27-residue Nt-CCR5 to form a helical structure and the helix formed by the peptide when bound to the gp120^{mi}CD4 complex suggests that this is the native conformation of the N-terminal segment of CCR5 bound to gp120/CD4.

In previous studies using the considerably shorter 14-residue Nt-CCR5 peptide the bound-structure was well defined for residues S7-Y15 and a helical structure was observed for I9-Y14³². In the present study the well-defined structure is twice as long (S7-I23) and the helical conformation spans a much larger region (P8-S17 and A20-I23). Moreover, previously no helical structure was observed for the short 14-residue Nt-CCR5(2-15) peptide when it was free in solution. These results indicate that both structural analyses and mapping of the binding determinant benefit from using a long peptide that includes all residues implicated in gp120 binding.

The orientation of the Nt-CCR5(1-27) peptide relative to gp120 and the target-cell membrane

A docking model for the gp120/Nt-CCR5(1-27) complex was calculated using the interacting residues on gp120 and Nt-CCR5(1-27) that were mapped in this investigation and by previous mutagenesis studies, but which did not include any information on pair-wise interactions in the complex. In this model, the C-terminal segment of the Nt-CCR5(1-27) continues towards the putative location of the target cell membrane. This is facilitated by the 90° bend formed at P19. The orientation suggested by the present study differs by approximately 180° from the proposed orientation of the 14-residue Nt-CCR5(2-15) peptide³². We note that the earlier docking calculation did not incorporate STD results or biochemical data in the calculation. Only after the model was determined it was examined to see whether it was consistent with the few STD interactions that had been assigned, those of the aromatic amino acids Y^S10, Y^S14 and Y15. Moreover, the larger number of residues (Y3, V5, P8, I9, Y^S10, D11, I12, Y^S14, Y15, T16, I23) used in the docking model with our 27-residue peptide allowed consideration of additional information. Therefore, it is not unreasonable that differences would be found in the two analyses.

In the present model CCR5^{Tys}14 occupies a binding pocket identical to that occupied in the previous model and to that occupied by Tys100c of 412d Fab in complex with gp120 and sCD4³². Also, similarly to the interaction of the other tyrosine sulfated residue (Tys100) in the 412d antibody binding site with gp120^{Arg}328, CCR5^{Tys}10 interacts with gp120^R328, although the distance between these residues is somewhat greater relative to the distances found in the crystal structure. This is despite our observation of similar strong STDs for both Tys10 and Tys14 upon irradiating the gp120^{mi}CD4 complex. Experimental data regarding pair-wise interactions between CCR5 and gp120 could improve the accuracy of the docking model. In the model calculated herein, CCR5^{Tyr}3 is located close to a pocket formed by the gp120-419^{RIKQI}423 segment which was previously identified by mutational analysis to bind sulfated Nt-CCR5 peptides^{12; 47}. No role for this gp120 segment in Nt-CCR5(2-15) binding was suggested in the previously published model³². The pocket formed by gp120-419^{RIKQI}423 exhibits striking similarity to the binding pocket for CCR5^{Tys}14 (and for Tys 100c of 412d Fab) which is also lined with two positively charged amino acids, gp120^{Arg}298 and gp120^{Arg}440, two isoleucine residues, gp120^{Ile}323 and gp120^{Ile}327, and polar residues, gp120^{Asn}302 and gp120^{Thr}303. Moreover, these two gp120 pockets resemble binding pockets for sulfotyrosine in SDF-1 and RANTES which are the natural ligands for CXCR4 and CCR5, respectively^{50; 51; 52}. This similarity in binding environment

suggests that the pocket formed by gp120-419^{RIKQI}⁴²³ could bind a sulfated tyrosine at position 3.

Affinity measurements of a triply tyrosine sulfated (at tyrosine residues 3, 10 and 14) Nt-CCR5 peptide to gp120 and its comparison to the binding affinity of a peptide sulfated at position 10 and 14 only to gp120, would reveal the role of CCR5^{Tys3} in gp120 binding if the measured affinity for the triply sulfated peptide is higher. However, one should bear in mind that the synthesis of a triply sulfated Nt-CCR5 is considerably more difficult than that of the homologous doubly sulfated peptide.

Implications for R5 to X4 phenotype conversion

R5 HIV-1 strains but not X4 HIV-1 strains recognize the CCR5 co-receptor. The course of HIV-1 infection is often characterized by a phenotype conversion from R5 to X4 virus. This conversion has been associated with mutation from a negatively charged residue to a positively charged residue at position 322 of gp120 despite the fact that no direct involvement of residue 322 in co-receptor interactions has been revealed⁵³. We previously found that a negative charge at position 322 is correlated with a positively charged residue at position 440⁵⁴.

The strong interaction of CCR5^{Tys14} with gp120^{Arg440} that is a major component of the binding pocket for tyrosine-sulfate in gp120 underlines the role of residue 440 in determining CCR5-tropism. The influence of residue 322 on the nature of the charge at position 440 explains the role of residue 322 on phenotype conversion. X4 strains lacking a positive charge at or near residue gp120⁴⁴⁰ would not bind to Nt-CCR5.

Conclusions

In conclusion, this study reveals new information on the structure of Nt-CCR5(1-27) while bound to the ^{tr}gp120/^{mi}CD4 complex, uncovers the existing helical tendency in the peptide in its free form and reports on residues of Nt-CCR5(1-27) that participate in complex binding. Moreover our study provides a plausible explanation for the role of residue 322 in phenotype conversion through its influence on the charge of the residue at position 440. Studies of peptides encompassing other extra-cellular segments of CCR5 and identification of pair-wise interactions between gp120 and CCR5-derived peptides will provide a detailed picture of the interface between the heptahelical CCR5 and the gp120/CD4 complex. Such knowledge is of fundamental interest and should be valuable in the ongoing struggle to control HIV-1.

MATERIALS AND METHODS

Synthesis of Nt-CCR5(1-27) [¹MDYQVSSPIY^{SO3H}DINY^{SO3H}YTSEPAQKINVKQ²⁷, Nt-CCR5¹⁻²⁷(C20A), Y10^{SO3H}, Y14^{SO3H}]

Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and resins were purchased from Advanced ChemTech (Louisville, KY) except for Fmoc-Tyr(SO₃H)-OH sodium salt, which was purchased from Bachem Inc. (Torrance, CA). Diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS) and all other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents used for synthesis and purification, were purchased from VWR Scientific (Piscataway, NJ) and Fisher Scientific (Springfield, NJ).

The fully protected Nt-CCR5(1-27) was synthesized using an automated peptide synthesizer (Applied Biosystems Model 433A) on 0.1 mmol of Fmoc-Gln(Trt)-Wang resin (0.6 mmol/g of substitution). The coupling strategy utilized FastMoc chemistry with the use of double

coupling for all amino acids except Fmoc-Tyr(SO₃H)-OH. Cleavage of the doubly sulfated peptide from the resin and the removal of the side chain protecting group were performed very carefully, using a mixture of TFA/TIS/H₂O (92/4/4) for 3 to 6 hr at 0°C, because of the pronounced acid-lability of the sulfated tyrosine. The progress of the reaction was monitored using HPLC. Under these conditions the hydrolysis of the tyrosine-O-sulfate ester should be sufficiently low to be acceptable for preparative purposes⁵⁵. The cleavage mixture was concentrated using a rotary evaporator, the peptide was precipitated with cold ether and isolated by centrifugation, and the precipitate was washed three times with ether and dried in vacuum. Crude product was purified by reverse phase HPLC (RP-HPLC) using a Delta Pak C₁₈ column (15 µm, 100 Å, 19 × 300 mm, Waters), the peptide was dissolved in the starting eluent and 10-20 mg of peptide were purified in each run using gradient elution: eluent A, 0.1M ammonium acetate buffer pH 6.8 in water; eluent B, acetonitrile; gradient 12-27% B over 60 min; flow rate, 5 ml/min; detection at 220 nm. The appropriate fractions were collected and lyophilized. Analytical reversed phase HPLC (RP-HPLC) was performed using an Eclipse XDB-C8 column (5 µm, 4.6 × 150 mm, Agilent) with either an ammonium acetate based buffer or TFA as the hydrophobic counter ion using gradient elution with either: eluent A, ammonium acetate buffer pH 6.8 in water; eluent B, acetonitrile; gradient 10-30% B over 20 min or eluent A, 0.025% TFA in water; eluent B, 0.025% TFA in acetonitrile; gradient 10-60% B over 20 min; detection at 220 nm. The yield of the final product (>95% homogeneity) was 12.3% (calculated on the basis of the loading of the resin). Mass spectrometric analysis using an ESI-MS instrument gave two molecular ion masses in positive scan of 3194 Da (the unsulfated derivative) and 3274 Da (the monosulfated derivative). In negative scan only a molecular ion mass of 3354 Da (theoretical value: 3355 Da) was observed. This result is consistent with what is reported in the literature and depends on the high lability of the sulfate ester in the gas phase.

Synthesis of Biotinylated Nt-CCR5(1-27)

[¹MDYQVSSPIY^{SO₃H}DINY^{SO₃H}YTSEPAQKINVVKQ²⁷GSGK^{Biotin}G]

The Nt-CCR5(1-27) analog biotinylated near the C-terminus was synthesized similarly using the 433A automated peptide synthesizer starting with a 167 mg, 0.6 mmol/g, 0.1 mmol Fmoc-Gly-Wang resin. A single coupling of Fmoc-Lys(Biotin)-OH was carried out using a 5-fold excess of this amino acid derivative. For the other amino acids, except for Fmoc-Tyr(SO₃H)-OH, standard double coupling with a 10-fold excess of the requisite amino acid was utilized. Cleavage from the resin was as above. The overall yield of the essentially homogeneous product was 11.7%. The mass determined by ESI-MS was 3968.2 (calc 3968.4).

Synthesis of the CD4 miniprotein

This miniprotein was synthesized following the literature procedure³⁴ and was pure by HPLC and ESI-MS.

Expression of gp120

A truncated⁸⁸⁻⁴⁹²gp120 lacking the V1 and V2 variable loops was expressed. To increase the affinity of the modified gp120_{JR-FL} molecule to CD4, two glycosylation sites were mutated and removed, N301Q and T388A (⁸⁸⁻⁴⁹²gp120ΔV1/V2(N301Q;T388A))⁵⁶.

The vector pSyn gp120, which encodes JF-RL gp120 and a leader sequence under the control of EF1α promoter, was provided by the NIH AIDS reagent program. The nucleotide sequence was optimized for expression in human cells and is GC rich. From this vector a gene encoding residues 88-483 of gp120 JR-FL was constructed and inserted into pIRES using polymerase chain reaction methodology. The pIRES vector was developed for high level expression of proteins in 293 human kidney cells and was provided by Dr. Kahana

(Weizmann Institute). It encodes the IgK secretion signal that enables the secretion of the gp120 protein to the growth medium and a 6× histidine tag followed by a Tobacco Etch Virus (tev) protease recognition site at the N-termini of gp120.

The gp120 was expressed in human embryonic kidney 293 cells lacking N-acetyl glucosaminyl transferase I enzymatic activity (GnTI- HEK293S cells). Proteins expressed in cells lacking this enzyme are homogeneously glycosylated with Man₅GlcNAc₂ glycans at sites normally occupied by complex or hybrid glycans⁵⁷. This construct yields a 56 kDa gp120 truncated molecule, hereafter referred to as glycosylated-^{tr}gp120. Treatment of this protein with Endo-H_F (New England Biolabs, MA, USA) removes the Man₅-GlcNAc carbohydrates leaving one GlcNAc attached to an asparagine residue of the protein, resulting in further reduction in molecular weight (~40 kDa). This protein is hereafter referred to as deglycosylated-^{tr}gp120.

Both ^{tr}gp120 constructs glycosylated and deglycosylated, were purified in three steps: first, by purification on a Cibacron Blue Sepharose column (GE Healthcare), which binds proteins through electrostatic and/or hydrophobic interactions with the aromatic anionic ligand; then by affinity chromatography using a nickel column (GE Healthcare); and finally, by gel filtration chromatography, using a superdex 200 column (GE Healthcare). These purification steps resulted in a protein yield of ~2-3 mg/liter and >95% purity. Identity of ^{tr}gp120 was verified by two means. First, by Western blot using the human anti-HIV-1 monoclonal antibody 447-52D which recognizes the V3 loop and second, by peptide finger-printing identification (MALDI-TOF).

Surface Plasmon Resonance (SPR) measurements

Binding affinities (K_D) were determined by SPR using a ProteOn XPR36 Protein Interaction Array System (Bio-Rad Haifa, Haifa, Israel)⁵⁸. All experiments were carried out at 300 K using SPR buffer (20 mM Tris, pH 7.0, 0.05% NaN₃, 0.005% Tween) with 300, 150 or 50 mM NaCl.

Biotinylated ^{mi}CD4 or biotinylated Nt-CCR5(1-27) was immobilized on a carboxymethylated dextran matrix chip, precoated with streptavidin (ProteOn NLC sensor chip). The immobilization procedure was allowed to continue until 424, 350 and 212 or 220 RU (response units; 1 RU = 1 pg of protein/mm²) of biotinylated Nt-CCR5(1-27) or biotinylated ^{mi}CD4, respectively, were attached to the streptavidin-precoated surface of the chip. A blank sensor surface without an immobilized peptide served as a negative control and as a reference for the binding interaction. Binding was then measured using serial dilutions of glycosylated and deglycosylated-^{tr}gp120 both complexed with ^{mi}CD4 or by itself in SPR buffer (concentration ranging from nano- to micromolar), which were simultaneously injected over the peptide-immobilized surface at a flow rate of 30 µl/min. Data were analyzed with the ProteOn Manager software after subtraction of a blank channel (negative control), using equilibrium analysis or a Langmuir 1:1 kinetic model.

Analytical Ultracentrifugation

Sedimentation equilibrium experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge (Beckman, Coulter, CA) at 300 K. Six-channel cells were used with an An60 titanium rotor. The cells were scanned using a step size of 0.001 cm, and data points were averaged over 10 replicates. Solutions of 10 µM ^{tr}gp120/^{mi}CD4 were prepared in Tris buffer pH 8.0 only or supplemented with 50 or 300 mM NaCl. Samples were initially scanned at 3,000 rpm to identify appropriate wavelengths for data collection. Data were collected at different wavelengths according to the sample's absorbance and the different rotation speed suitable for the molecular mass of possible multimerization states (i.e.,

monomers, dimers and trimers) as suggested by the manufacturer's manual. Equilibrium was reached after 10 hr and verified by overlay and subtraction of at least two successive scans.

Weight-averaged molecular masses were obtained by averaging the molecular masses from all data sets after reaching equilibrium. The apparent molecular mass was calculated by the formula $M = [2RT/(1 - \nu\rho)\omega^2][d(\ln \text{Abs})/dr^2]$, where M is the solute molar weight (in grams per mole), R is the gas constant in grams per square centimeter per square minute, T is the temperature in Kelvin, $1 - \nu\rho = 0.27$ (ν is the partial specific volume in milliliter per gram; ρ is the density in grams per milliliter)⁵⁹, ω is the angular velocity of the rotor in revolutions per minute, and $d(\ln \text{Abs})/dr^2$ is the first derivative of $\ln \text{Abs}$ with respect to r^2 , where Abs is the absorbance of the solute (OD) at r which is the radial distance from the axis of rotation⁶⁰. Residuals, which are the difference between each experimental data point and the corresponding point on the curve calculated from the model equation, were calculated. A random distribution of residuals points about the zero value indicates a good fit, whereas systematic errors are characteristic of association and non-ideality.

NMR Sample Preparation

Samples of the free peptide contained 0.6 mM Nt-CCR5(1-27) dissolved in a 20 mM aqueous solution of D₁₁-Tris, pH 7.0, containing 300 mM NaCl, 1 mM D₁₆-EDTA, 95% H₂O, 5% D₂O and 0.05% NaN₃ or 1.25 mM Nt-CCR5(1-27) dissolved in 10 mM aqueous solution of phosphate buffer, pH 7.0, 95% H₂O, 5% D₂O and 0.05% NaN₃. Solution of deglycosylated-^{tr}gp120/^{mi}CD4 and Nt-CCR5(1-27) in a 20 mM aqueous solution of D₁₁-Tris, pH 7.0, containing 300 mM NaCl, 1 mM D₁₆-EDTA, 95% H₂O, 5% D₂O and 0.05% NaN₃ was prepared. The final concentrations of deglycosylated-^{tr}gp120/^{mi}CD4 and Nt-CCR5(1-27) were either 0.1 mM, and 1.0 mM, respectively (1:10) for the transferred NOE measurements or 0.15 mM, and 0.1 mM, respectively (1:0.7) for the T_{1ρ} measurements (See below). Concentrations were determined using UV absorbance measurement at 280 nm.

NMR Measurements

All NMR spectra were measured on a Bruker DRX 800 MHz spectrometer equipped with a cryo-probe. Water suppression was achieved using the WATERGATE (WATER suppression by Gradient-Tailored Excitation) sequence^{61; 62} or excitation sculpting with gradients⁶³.

2D HOHAHA and T_{1ρ}-filtered NOESY⁶⁴ spectra of the deglycosylated-^{tr}gp120/^{mi}CD4/Nt-CCR5(1-27) complex were acquired with mixing times of 120 and 100 ms, respectively, at 310, 300, 288 and 274 K. A 15 ms spin-lock pulse length was used in the T_{1ρ}-filtered NOESY to ensure that all of the broad proton resonances of the deglycosylated-^{tr}gp120/^{mi}CD4 vanish⁶⁴.

STD^{41; 42} spectra were acquired using an on-resonance irradiation of the protein at a chemical shift of -2.0 (where no peptide resonances are found), an off-resonance irradiation applied at 30 ppm (where no protein signals are present) and a total saturation time of 2.0 s at 300 and 308 K. The protein presaturation was achieved by a train of Gauss-shaped pulses of 500 ms length each, separated by a 1 ms delay. 2D-STD-HOHAHA spectra were acquired with 40 and 120 ms mixing times. 2D HOHAHA spectra of the free Nt-CCR5(1-27) were acquired using mixing times of 120 ms and NOESY spectra were acquired using mixing times of 100 and 200 ms. Both HOHAHA and NOESY experiments were carried out at 300, 284 and 274 K. Sequential assignment of Nt-CCR5(1-27) was performed according to well-established procedures⁶⁵. Data were processed and analyzed using the Topspin (Bruker-De), NMRPipe⁶⁶ and NMRView software^{67; 68}.

$T_{1\rho}$ -filtered HOHAHA experiments incorporated a spin-lock pulse after the initial 90° pulse, with varying durations between 0 and 200 ms in 40 ms increments. These spectra were acquired with a mixing time of 80 ms and were carried out at 288 K. Proton $T_{1\rho}$ values were determined by extracting peak intensities of H_N-H_α peaks (I) and fitting the relaxation data to a single exponential decay, $I(t)=I_0\cdot\exp(-t/T_{1\rho})$, where I_0 and $I(t)$ are the peak intensities with spin-lock length 0 ms or other, respectively. This method was developed previously and applied to study the relaxation times of the residues of an acetylcholine receptor peptide bound to bungarotoxin⁴³.

$^3J_{HNH\alpha}$ -coupling constant values were estimated from the HOHAHA spectrum acquired at 274 K using 8K data points in F2 (zero-filled to 16K) and 800 increments in the F1 dimension. The AURELIA software package (Bruker-De)⁶⁹ was used to measure the coupling constant values. C_α and C_β chemical shift values of the free peptide were measured by acquiring a 2D 1H - ^{13}C -HSQC spectrum, using ^{13}C natural abundance.

Structure Calculation

The structure of bound Nt-CCR5(1-27) was calculated with the Crystallography and NMR System (CNS) program using the hybrid distance-geometry - dynamical simulated-annealing method⁷⁰. The calculation started from an extended conformation. NOE constraints, obtained from the NOESY experiment, and dihedral angles constraints, determined using the $^3J_{HNH\alpha}$ -couplings, were given as the input parameters. The NOE constraints were calculated from peak intensities. Distances were calibrated using the r^6 function. Median NOE intensity corresponded to a distance of 2.7 Å and were verified using the $H\delta(i)/H\epsilon(i)$ NOE of tyrosine residues, which corresponds to 2.5 Å. Each NOE constraint was assigned with a lower limit of 1.8 Å and an upper limit of 130% of the calculated distance, in order to account for internal motion and proton multiplicity⁷¹. The Φ dihedral angles along the polypeptide backbone were constrained to $-65 \pm 25^\circ$ ⁷¹ for all of the amino acids exhibiting $^3J_{HNH\alpha} < 6$ Hz. In transferred-NOESY experiments, free peptide resonances are detected and therefore no measurement of $^3J_{HNH\alpha}$ values for the bound peptide was possible. However, the observed medium-range NOE interactions indicate that any helical propensity in the free peptide is reinforced upon binding the gp120/miCD4 complex. Therefore, $^3J_{HNH\alpha}$ values that were determined for the free peptide were used in structure calculation. Initially no hydrogen bonds were used as constraints for structure calculation. During the refinement process hydrogen bond constraints were gradually introduced into the calculation if a hydrogen bond was present in 50% or more of the calculated structures as analyzed by MOLMOL³⁹. Fifty structures with no violations of the NMR constraints larger than 0.5 Å were generated (out of which the 10 lowest energy structures were used for analysis).

Model Generation

The Model of Nt-CCR5(1-27) bound to gp120 was calculated using the HADDOCK web-server⁴⁶. Parameters for docking calculations were based on mutagenesis and crystallographic data previously reported for gp120^{12; 32; 47} and STD and $T_{1\rho}$ data for Nt-CCR5(1-27) as well as mutagenetic analysis previously reported for Nt-CCR5^{21; 22; 33; 48}. Starting structures for the docking were the previously published crystal structure of gp120, which was solved in complex with a tyrosine-sulfated antibody [PDB entry 2QAD; chain A³²] and the average structure of bound Nt-CCR5(1-27), calculated in the course of this study. A total of 1000 structures were calculated in the rigid body minimization. Semi-flexible simulated annealing followed by refinement in explicit water was performed for the best 200 solutions based on the HADDOCK score (weighted sum of all the energy terms and the buried surface area). The calculated models were clustered using a 7.5 Å interface RMSD cutoff. Hydrogen bonds within the modeled complex were calculated by the find-

hbonds program ⁷², which uses criteria for hydrogen bonds adapted from Levitt and co-workers. Salt bridges were determined between residues with opposite charges closer than 5 Å, and van-der-Waals interactions between hydrogen atoms closer than 4 Å.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Profs. Prashen Chelikani and H. Gobind Khorana for kindly providing the GnTI-HEK293S cells, Prof. Zigi Kahana for supplying the pIRES vector and Prof. Gideon Schreiber and Mr. Ori Cohavi for allowing the use of the Proteon. Useful comments and suggestions of Prof. Miriam Eisenstein are gratefully acknowledged.

Abbreviations

AIDS	acquired immune deficiency syndrome
CCR5	CC chemokine receptor 5
CXCR4	CXC chemokine receptor 4
DIEA	Diisopropylethylamine
DMF	Dimethylformamide
Fmoc	Fluorenylmethyloxycarbonyl
FPLC	fast protein liquid chromatography
HIV-1	human immunodeficiency virus type 1
HOHAHA	homonuclear Hartmann-Hahn spectroscopy
HPLC	high-pressure liquid-chromatography
HSQC	heteronuclear single-quantum correlation
K_D	equilibrium dissociation constant
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
SPR	surface plasmon resonance
STD	saturation transfer difference
TFA	trifluoroacetic acid
TIS	triisopropylsilane
Tys	sulfotyrosine

REFERENCES

1. Freed EO, Martin MA. The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection. *J. Biol. Chem.* 1995; 270:23883–23886. [PubMed: 7592573]
2. Trkola A, Dragic T, Arthos J, Binley JM, Olson WC, Allaway GP, Cheng-Mayer C, Robinson J, Maddon PJ, Moore JP. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature.* 1996; 384:184–187. [PubMed: 8906796]
3. Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, Borsetti A, Cardoso AA, Desjardin E, Newman W, Gerard C, Sodroski J. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature.* 1996; 384:179–183. [PubMed: 8906795]

4. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*. 1996; 272:872–877. [PubMed: 8629022]
5. Chan DC, Kim PS. HIV entry and its inhibition. *Cell*. 1998; 93:681–684. [PubMed: 9630213]
6. Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, Hendrickson WA, Sodroski J. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science*. 1998; 280:1949–1953. [PubMed: 9632396]
7. Cormier JW, Rivolta I, Tateyama M, Yang AS, Kass RS. Secondary structure of the human cardiac Na⁺ channel C terminus: evidence for a role of helical structures in modulation of channel inactivation. *J. Biol. Chem*. 2002; 277:9233–9241. [PubMed: 11741959]
8. Wu X, Sambor A, Nason MC, Yang ZY, Wu L, Zolla-Pazner S, Nabel GJ, Mascola JR. Soluble CD4 broadens neutralization of V3-directed monoclonal antibodies and guinea pig vaccine sera against HIV-1 subtype B and C reference viruses. *Virology*. 2008; 380:285–295. [PubMed: 18804254]
9. Tugarinov V, Zvi A, Levy R, Hayek Y, Matsushita S, Anglister J. NMR structure of an anti-gp120 antibody complex with a V3 peptide reveals a surface important for co-receptor binding. *Structure*. 2000; 8:385–395. [PubMed: 10801487]
10. Sharon M, Kessler N, Levy R, Zolla-Pazner S, Gorlach M, Anglister J. Alternative conformations of HIV-1 V3 loops mimic beta hairpins in chemokines, suggesting a mechanism for coreceptor selectivity. *Structure (Camb)*. 2003; 11:225–236. [PubMed: 12575942]
11. Rosen O, Chill J, Sharon M, Kessler N, Mester B, Zolla-Pazner S, Anglister J. Induced fit in HIV-neutralizing antibody complexes: evidence for alternative conformations of the gp120 V3 loop and the molecular basis for broad neutralization. *Biochemistry*. 2005; 44:7250–7258. [PubMed: 15882063]
12. Cormier EG, Dragic T. The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor. *J. Virol*. 2002; 76:8953–8957. [PubMed: 12163614]
13. Mueller A, Strange PG. The chemokine receptor, CCR5. *Int. J. Biochem. Cell Biol*. 2004; 36:35–38. [PubMed: 14592532]
14. Lusso P. HIV and chemokines: implications for therapy and vaccine. *Vaccine*. 2002; 20:1964–1967. [PubMed: 11983255]
15. Clapham PR, McKnight A. HIV-1 receptors and cell tropism. *Br. Med. Bull*. 2001; 58:43–59. [PubMed: 11714623]
16. Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, Buchbinder SP, Vittinghoff E, Gomperts E, Donfield S, Vlahov D, Kaslow R, Saah A, Rinaldo C, Detels R, O'Brien SJ. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science*. 1996; 273:1856–1862. [PubMed: 8791590]
17. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*. 1996; 86:367–377. [PubMed: 8756719]
18. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature*. 1996; 382:722–725. [PubMed: 8751444]
19. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dautet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science*. 1983; 220:868–871. [PubMed: 6189183]
20. Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, Macartney M, Mori J, Rickett G, Smith-Burchnell C, Napier C, Webster R, Armour D, Price D, Stammen B, Wood A, Perros M. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine

- receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob. Agents Chemother.* 2005; 49:4721–4732. [PubMed: 16251317]
21. Dragic T, Trkola A, Lin SW, Nagashima KA, Kajumo F, Zhao L, Olson WC, Wu L, Mackay CR, Allaway GP, Sakmar TP, Moore JP, Maddon PJ. Amino-terminal substitutions in the CCR5 coreceptor impair gp120 binding and human immunodeficiency virus type 1 entry. *J. Virol.* 1998; 72:279–285. [PubMed: 9420225]
 22. Farzan M, Choe H, Vaca L, Martin K, Sun Y, Desjardins E, Ruffing N, Wu L, Wyatt R, Gerard N, Gerard C, Sodroski J. A tyrosine-rich region in the N terminus of CCR5 is important for human immunodeficiency virus type 1 entry and mediates an association between gp120 and CCR5. *J. Virol.* 1998; 72:1160–1164. [PubMed: 9445013]
 23. Lee B, Sharron M, Blanpain C, Doranz BJ, Vakili J, Setoh P, Berg E, Liu G, Guy HR, Durell SR, Parmentier M, Chang CN, Price K, Tsang M, Doms RW. Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *J. Biol. Chem.* 1999; 274:9617–9626. [PubMed: 10092648]
 24. Samson M, LaRosa G, Libert F, Paindavoine P, Detheux M, Vassart G, Parmentier M. The Second Extracellular Loop of CCR5 Is the Major Determinant of Ligand Specificity. *J. Biol. Chem.* 1997; 272:24934–24941. [PubMed: 9312096]
 25. Blanpain C, Migeotte I, Lee B, Vakili J, Doranz BJ, Govaerts C, Vassart G, Doms RW, Parmentier M. CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. *Blood.* 1999; 94:1899–1905. [PubMed: 10477718]
 26. Farzan M, Vasilieva N, Schnitzler CE, Chung S, Robinson J, Gerard NP, Gerard C, Choe H, Sodroski J. A tyrosine-sulfated peptide based on the N terminus of CCR5 interacts with a CD4-enhanced epitope of the HIV-1 gp120 envelope glycoprotein and inhibits HIV-1 entry. *J. Biol. Chem.* 2000; 275:33516–33521. [PubMed: 10938094]
 27. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, Gerard NP, Gerard C, Sodroski J, Choe H. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell.* 1999; 96:667–676. [PubMed: 10089882]
 28. Cormier EG, Persuh M, Thompson DA, Lin SW, Sakmar TP, Olson WC, Dragic T. Specific interaction of CCR5 amino-terminal domain peptides containing sulfotyrosines with HIV-1 envelope glycoprotein gp120. *Proc. Natl. Acad. Sci. U S A.* 2000; 97:5762–5767. [PubMed: 10823934]
 29. Choe H, Martin KA, Farzan M, Sodroski J, Gerard NP, Gerard C. Structural interactions between chemokine receptors, gp120 Env and CD4. *Semin. Immunol.* 1998; 10:249–257. [PubMed: 9653051]
 30. Brower ET, Schon A, Klein JC, Freire E. Binding thermodynamics of the N-terminal peptide of the CCR5 coreceptor to HIV-1 envelope glycoprotein gp120. *Biochemistry.* 2009; 48:779–785. [PubMed: 19170639]
 31. Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V, Stevens RC. Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science.* 2010; 330:1066–1071. [PubMed: 20929726]
 32. Huang CC, Lam SN, Acharya P, Tang M, Xiang SH, Hussan SS, Stanfield RL, Robinson J, Sodroski J, Wilson IA, Wyatt R, Bewley CA, Kwong PD. Structures of the CCR5 N terminus and of a tyrosine-sulfated antibody with HIV-1 gp120 and CD4. *Science.* 2007; 317:1930–1934. [PubMed: 17901336]
 33. Rabut GE, Konner JA, Kajumo F, Moore JP, Dragic T. Alanine substitutions of polar and nonpolar residues in the amino-terminal domain of CCR5 differently impair entry of macrophage- and dualtropic isolates of human immunodeficiency virus type 1. *J. Virol.* 1998; 72:3464–3468. [PubMed: 9525683]
 34. Martin L, Stricher F, Misse D, Sironi F, Pugniere M, Barthe P, Prado-Gotor R, Freulon I, Magne X, Roumestand C, Menez A, Lusso P, Veas F, Vita C. Rational design of a CD4 mimic that inhibits HIV-1 entry and exposes cryptic neutralization epitopes. *Nat. Biotechnol.* 2003; 21:71–76. [PubMed: 12483221]
 35. Huang CC, Stricher F, Martin L, Decker JM, Majeed S, Barthe P, Hendrickson WA, Robinson J, Roumestand C, Sodroski J, Wyatt R, Shaw GM, Vita C, Kwong PD. Scorpion-toxin mimics of

- CD4 in complex with human immunodeficiency virus gp120 crystal structures, molecular mimicry, and neutralization breadth. *Structure*. 2005; 13:755–768. [PubMed: 15893666]
36. Howlett GJ, Minton AP, Rivas G. Analytical ultracentrifugation for the study of protein association and assembly. *Curr. Opin. Chem. Biol.* 2006; 10:430–436. [PubMed: 16935549]
 37. Myszka DG, Sweet RW, Hensley P, Brigham-Burke M, Kwong PD, Hendrickson WA, Wyatt R, Sodroski J, Doyle ML. Energetics of the HIV gp120-CD4 binding reaction. *Proc. Natl. Acad. Sci. U S A.* 2000; 97:9026–9031. [PubMed: 10922058]
 38. Mechulam A, Cerutti M, Pugniere M, Misse D, Gajardo J, Roquet F, Robinson J, Veas F. Highly conserved beta16/beta17 beta-hairpin structure in human immunodeficiency virus type 1 YU2 gp120 is critical for CCR5 binding. *J. Mol. Med.* 2005; 83:542–552. [PubMed: 15905975]
 39. Koradi R, Billeter M, Wuthrich K. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* 1996; 14:51–55. 29–32. [PubMed: 8744573]
 40. Kabsch W, Sander C. Dictionnary of protein secondary structure : Pattern recognition of hydrogen bonded and geometrical features. *Biopolymers.* 1983; 22:2577–2637. [PubMed: 6667333]
 41. Mayer M, Meyer B. Characterization of Ligand Binding by Saturation Transfer Difference NMR Spectroscopy. *Angew. Chem. Int. Ed.* 1999; 38:1784–1788.
 42. Mayer M, Meyer B. Group epitope mapping by saturation transfer difference NMR to identify segments of a ligand in direct contact with a protein receptor. *J. Am. Chem. Soc.* 2001; 123:6108–6117. [PubMed: 11414845]
 43. Samson AO, Chill JH, Anglister J. Two-dimensional measurement of proton T1rho relaxation in unlabeled proteins: mobility changes in alpha-bungarotoxin upon binding of an acetylcholine receptor peptide. *Biochemistry.* 2005; 44:10926–10934. [PubMed: 16086595]
 44. Cheetham JC, Redfield C, Griest RE, Raleigh DP, Dobson CM, Rees AR. Use of two-dimensional 1H nuclear magnetic resonance to study high-affinity antibody-peptide interactions. *Methods Enzymol.* 1991; 203:202–228. [PubMed: 1762556]
 45. Zvi A, Kustanovich I, Feigelson D, Levy R, Eisenstein M, Matsushita S, Richalet-Secordel P, Regenmortel MH, Anglister J. NMR mapping of the antigenic determinant recognized by an anti-gp120, human immunodeficiency virus neutralizing antibody. *Eur. J. Biochem.* 1995; 229:178–187. [PubMed: 7538073]
 46. de Vries SJ, van Dijk M, Bonvin AM. The HADDOCK web server for data-driven biomolecular docking. *Nat. Protoc.* 2010; 5:883–897. [PubMed: 20431534]
 47. Cormier EG, Tran DN, Yukhayeva L, Olson WC, Dragic T. Mapping the determinants of the CCR5 amino-terminal sulfopeptide interaction with soluble human immunodeficiency virus type 1 gp120-CD4 complexes. *J. Virol.* 2001; 75:5541–5549. [PubMed: 11356961]
 48. Blanpain C, Doranz BJ, Vakili J, Rucker J, Govaerts C, Baik SS, Lorthioir O, Migeotte I, Libert F, Baleux F, Vassart G, Doms RW, Parmentier M. Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 Env protein. *J. Biol. Chem.* 1999; 274:34719–34727. [PubMed: 10574939]
 49. Lam SN, Acharya P, Wyatt R, Kwong PD, Bewley CA. Tyrosine-sulfate isosteres of CCR5 N-terminus as tools for studying HIV-1 entry. *Bioorg. Med. Chem.* 2008; 16:10113–10120. [PubMed: 18952441]
 50. Duma L, Haussinger D, Rogowski M, Lusso P, Grzesiek S. Recognition of RANTES by extracellular parts of the CCR5 receptor. *J. Mol. Biol.* 2007; 365:1063–1075. [PubMed: 17101151]
 51. Farzan M, Babcock GJ, Vasilieva N, Wright PL, Kiprilov E, Mirzabekov T, Choe H. The role of post-translational modifications of the CXCR4 amino terminus in stromal-derived factor 1 alpha association and HIV-1 entry. *J. Biol. Chem.* 2002; 277:29484–29489. [PubMed: 12034737]
 52. Veldkamp CT, Seibert C, Peterson FC, Sakmar TP, Volkman BF. Recognition of a CXCR4 sulfotyrosine by the chemokine stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12). *J. Mol. Biol.* 2006; 359:1400–1409. [PubMed: 16725153]
 53. De Jong JJ, De Ronde A, Keulen W, Tersmette M, Goudsmit J. Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. *J. Virol.* 1992; 66:6777–6780. [PubMed: 1404617]

54. Rosen O, Samson AO, Anglister J. Correlated mutations at gp120 positions 322 and 440: implications for gp120 structure. *Proteins*. 2008; 71:1066–1070. [PubMed: 18275085]
55. Yagami T, Shiwa S, Futaki S, Kitagawa K. Evaluation of the final deprotection system for the solid-phase synthesis of Tyr(SO₃H)-containing peptides with 9-fluorenylmethyloxycarbonyl (Fmoc)-strategy and its application to the synthesis of cholecystokinin (CCK)-12. *Chem. Pharm. Bull. (Tokyo)*. 1993; 41:376–380. [PubMed: 8500203]
56. Koch M, Pancera M, Kwong PD, Kolchinsky P, Grundner C, Wang L, Hendrickson WA, Sodroski J, Wyatt R. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. *Virology*. 2003; 313:387–400. [PubMed: 12954207]
57. Reeves PJ, Callewaert N, Contreras R, Khorana HG. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. *Proc. Natl. Acad. Sci. U S A*. 2002; 99:13419–13424. [PubMed: 12370423]
58. Bravman T, Bronner V, Lavie K, Notcovich A, Papalia GA, Myszka DG. Exploring “one-shot” kinetics and small molecule analysis using the ProteOn XPR36 array biosensor. *Anal. Biochem*. 2006; 358:281–288. [PubMed: 16962556]
59. Biron Z, Khare S, Quadt SR, Hayek Y, Naider F, Anglister J. The 2F5 Epitope Is Helical in the HIV-1 Entry Inhibitor T-20. *Biochemistry*. 2005; 44:13602–13611. [PubMed: 16216084]
60. Chun PW, Kim SJ. Determination of equilibrium constants of associating protein systems. Graphical analysis for discrete and indefinite association. *Biochemistry*. 1970; 9:1957–1961. [PubMed: 5442164]
61. Piotto M, Saudek V, Sklenar V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J. Biomol. NMR*. 1992; 2:661–665. [PubMed: 1490109]
62. Sklenar V, Piotto M, Leppik R, Saudek V. Gradient-Tailored Water Suppression For H-1-N-15 Hsqc Experiments Optimized to Retain Full Sensitivity. *J. Magn. Reson. Ser. A*. 1993; 102:241–245.
63. Hwang T-L, Shaka AJ. Water suppression that works. Excitation sculpting using arbitrary waveforms and pulsed field gradients. *J. Magn. Reson. Ser. A*. 1995; 112:275–279.
64. Scherf T, Anglister J. A T1 rho-filtered two-dimensional transferred NOE spectrum for studying antibody interactions with peptide antigens. *Biophys. J*. 1993; 64:754–761. [PubMed: 8386014]
65. Wuthrich, K. *NMR of Proteins and Nucleic Acids*. Wiley; New York: 1986.
66. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR*. 1995; 6:277–293. [PubMed: 8520220]
67. Johnson BA, Blevins RA. Nmr View - a Computer-Program for the Visualization and Analysis of Nmr Data. *J. Biomol. NMR*. 1994; 4:603–614.
68. Johnson BA. Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol. Biol*. 2004; 278:313–352. [PubMed: 15318002]
69. Neidig K-P, Geyer M, Gorler A, Antz C, Saffrich R, Beneicke W, Kalbitzer H. AURELIA, a program for computer-aided analysis of multidimensional NMR spectra. *J. Biomol. NMR*. 1995; 6:255–270.
70. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D. Biol. Crystallogr*. 1998; 54(Pt 5):905–921. [PubMed: 9757107]
71. Roberts, GCK. *NMR of macromolecules*. Rickwood, D.; Hames, BD., editors. Oxford University Press; New York: 1993.
72. Gerstein M. A Resolution-Sensitive Procedure for Comparing Protein Surfaces and its Application to the Comparison of Antigen-Combining Sites. *Acta Cryst*. 1992; A48:271–276.

Fig. 1. NMR spectra of free Nt-CCR5(1-27)

Sections of (A) NOESY spectrum at 300 K, (B) NOESY spectrum at 274 K. Experiments were carried out on a 1.25 mM Nt-CCR5(1-27) sample (10 mM phosphate buffer, pH 7.0 with no sodium chloride added), using 250 and 200 ms mixing times for spectra at 300 and 274 K, respectively. Spectra were measured on an 800 MHz NMR spectrometer equipped with a cryo-probe. A few example interactions are labeled.

Fig. 2. Medium-range NOE interactions of free and gp120-bound Nt-CCR5(1-27)

NOE interactions of (a) derived from $T_{1\rho}$ -filtered NOESY spectra of 1 mM of Nt-CCR5(1-27) in the presence of 0.1 mM deglycosylated- 125 I-gp120/ 125 I-miCD4 complex measured at 274, 288, 300 and 308 K, in order to resolve peak overlap, in 20 mM D_{11} -Tris buffer, 300 mM NaCl, pH 7.0 and (b) free Nt-CCR5(1-27) derived from a NOESY spectrum measured at 274 K in 10 mM phosphate buffer, pH 7.0 with no sodium chloride added. The observed inter-residue connectivities are marked with filled rectangles, and the connectivities that could not be unambiguously assigned due to cross peak overlaps are marked with empty rectangles. All spectra were measured on an 800 MHz NMR spectrometer equipped with a cryo-probe.

Fig. 3. NMR spectra of bound (a) and free (b) Nt-CCR5(1-27)

(a) A section of a $T_{1\rho}$ -filtered NOESY spectrum of Nt-CCR5(1-27) and deglycosylated- Δ gp120/ Δ miCD4; Experiment carried out on a 1 mM Nt-CCR5(1-27) sample in the presence of 0.1 mM deglycosylated- Δ gp120/ Δ miCD4 (10:1). (b) A section of a NOESY spectrum of free Nt-CCR5(1-27); Experiment carried out on a 0.6 mM Nt-CCR5(1-27) sample. Both experiments were carried out on samples dissolved in the same conditions (20 mM D_{11} -Tris buffer, 300 mM NaCl, pH 7.0) using 100 ms mixing time and were measured at 300 K on an 800 MHz NMR spectrometer equipped with a cryo-probe. A few example interactions are labeled. *Peak assignment is better resolved at a different temperature.

Fig. 4. Ensemble of 10 lowest energy structures of Bound Nt-CCR5(1-27)
The converging structures of the segment S7-I23 of Nt-CCR5(1-27) bound to deglycosylated-^{tr}gp120/^{mi}CD4 are presented.

Fig. 5. 1D-STD NMR spectrum of Nt-CCR5(1-27) in the presence of deglycosylated-trgp120/miCD4

Reference and difference spectra are shown in black and red, respectively. (a) Aromatic region showing assignment. (b) Amide, aromatic and aliphatic regions showing identifiable assignment of different residues. Spectra were recorded at 300 K on samples containing 0.1 mM deglycosylated-trgp120/miCD4 and 1 mM Nt-CCR5(1-27) in 20 mM D₁₁-Tris buffer, 300 mM NaCl, pH 7.0 at pH 7.0 using on-resonance irradiation at -2 ppm. Superscript S indicates sulfated Tyr.

Fig. 6. HOHAHA and 2D-STD-HOHAHA spectra

The aliphatic (a,b) and aromatic (c,d) region of a HOHAHA spectrum (a,c) and a 2D-STD-HOHAHA spectrum acquired using on-resonance irradiation at -2 ppm (b,d) of 1 mM Nt-CCR5(1-27) in the presence of 0.1 mM deglycosylated-^{tr}gp120/^{mi}CD4 in 20 mM D₁₁-Tris buffer, 300 mM NaCl, pH 7.0. Arrows and dotted lines show assignments. Spectra were measured at 308 K on an 800 MHz NMR spectrometer equipped with a cryo-probe.

Fig. 7. Quantification of STD enhancements

1D-STD (grey bars) and 2D-STD-HOHAHA (black bars) enhancements calculated by dividing peak intensities in the 1D-STD or 2D-STD-HOHAHA to the corresponding peak intensities in a 1D or HOHAHA spectra (respectively). Values were normalized to the largest enhancement. Bars represent the highest enhancement that could be unambiguously determined for each residue. Asterisk marks residues whose resonances could be resolved in the 1D-STD spectrum and for which quantification of the STD enhancement could be determined but in the 2D-STD, cross-peak overlap due to lower resolution in the acquisition dimension of the 2D-spectrum in comparison with the 1D-STD spectrum, prevented the quantification of the STD for each of the residues whose cross-peaks overlapped (i.e. Y3 and Y15, V5 and V25, I12 and I23).

Fig. 8. Changes in the $T_{1\rho}$ relaxation times of Nt-CCR5(1-27) residues upon binding to the trgp120/miCD4 complex

Changes in $T_{1\rho}$ relaxation times of H_α of various residues in Nt-CCR5(1-27), presented as the ratio (percentage) between the $T_{1\rho}$ relaxation time of the Nt-CCR5(1-27) residue bound to deglycosylated- trgp120/miCD4 and that of the same residue in the free form, as a function of residue number. Only residues which were well resolved in the HOHAHA spectra are presented. A 60% value of the ratio representing a reduction of 40% in $T_{1\rho}$, is drawn as cutoff. Residues exhibiting ratio above this cut-off are regarded as residues that do not interact with the deglycosylated- trgp120/miCD4 complex.

Fig. 9. Docking model of Nt-CCR5(1-27) bound to gp120

The lowest energy model of the Nt-CCR5(1-27)/gp120 complex within the highest score HADDOCK cluster is presented. The putative location of the membrane with respect to the Nt-CCR5(1-27) is drawn. The C4 and V3 regions of gp120 are depicted in blue and cyan, respectively, and Nt-CCR5(1-27) in red. Binding residues are depicted in stick representation and labeled.

Fig. 10. Electrostatic potential of the gp120 surface

(a) Vacuum electrostatic potential of gp120. The C4 and V3 regions of gp120 are depicted in cartoon representation and binding residues are depicted in stick representation and labeled. (b) A helical wheel projection obtained by looking down the axis of the P8-E18 segment of the calculated Nt-CCR5 structure. C α atoms are depicted as balls. The projection shows a negatively charged face which interacts with the positively charged face of gp120.

Table 1

NMR constraints and structural statistics for the 10 lowest energy structures of Nt-CCR5(1-27) peptide bound to deglycosylated-^{tr}gp120/^{mi}CD4

Total distance constraints	401
Intra-residue	214
Sequential	101
Medium	86
Long range	0
Φ dihedral angle	21
Hydrogen bond constraints	3
Mean RMSD values (Å), (residues P8-S17)	
Backbone atoms	0.6 ± 0.3
Heavy atoms	1.6 ± 0.5
Mean RMSD values (Å), (residues S7-I23)	
Backbone atoms	1.5 ± 0.6
Heavy atoms	2.4 ± 0.7
Mean RMSD values (Å), (residues M1-Q27)	
Backbone atoms	5.1 ± 1.6
Heavy atoms	6.1 ± 1.6

Table 2Nt-CCR5(1-27)/gp120 docking statistics and interface interactions analysis^a

gp120 active residues	R298, N301, N302, T303, R304, K305, I323, I324, G325, D326, I327, R328, R419, I420, K421, Q422, R440 and R444		
Nt-CCR5(1-27) active residues	D2, Y3, V5, P8, I9, Y ^S 10, D11, N13, I12, Y ^S 14, Y15, T16, S17, E18, Q21, K22 and I23		
Haddock Score	-120.7 +/- 22.1		
Haddock Cluster size	58		
Buried Surface Area	1826.2 +/- 102.6		
RMSD from the overall lowest-energy structure	1.3 +/- 0.7 Å		
I	<u>Hydrogen bonds:</u>	<u>Salt bridges:</u>	<u>Van der waals:</u>
n	gp120 ^{R328} - CCR5 ^{P8}	gp120 ^{R328} - CCR5 ^{D11}	gp120 ^{R419} - CCR5 ^{Y3}
t	gp120 ^{R298} - CCR5 ^{Y^S14}	gp120 ^{R298} - CCR5 ^{Y^S14}	gp120 ^{I423} - CCR5 ^{P8}
e	gp120 ^{N300} - CCR5 ^{Y^S14}	gp120 ^{R440} - CCR5 ^{Y^S14}	gp120 ^{R328} - CCR5 ^{I9}
r	gp120 ^{N302} - CCR5 ^{Y^S14}	gp120 ^{K305} - CCR5 ^{E18}	gp120 ^{R328} - CCR5 ^{Y^S10}
a	gp120 ^{T303} - CCR5 ^{Y^S14}	gp120 ^{R440} - CCR5 ^{E18}	gp120 ^{I327} - CCR5 ^{I12}
c	gp120 ^{R440} - CCR5 ^{Y^S14}	gp120 ^{E322} - CCR5 ^{K22}	gp120 ^{R328} - CCR5 ^{I12}
t	gp120 ^{I323} - CCR5 ^{Y15}		gp120 ^{T303} - CCR5 ^{Y^S14}
i	gp120 ^{K305} - CCR5 ^{E18}		gp120 ^{I323} - CCR5 ^{Y^S14}
o	gp120 ^{R440} - CCR5 ^{E18}		gp120 ^{I327} - CCR5 ^{Y^S14}
n			gp120 ^{I323} - CCR5 ^{Y15}
s			gp120 ^{I324} - CCR5 ^{Y15}

^aSuperscript S denotes Tys