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Engineering the metamorphic chemokine Lymphotactin/XCL1 into the GAG-binding, HIV-inhibitory dimer conformation

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Abstract

Unlike other chemokines, XCL1 undergoes a distinct metamorphic interconversion between a canonical monomeric chemokine fold and a unique β -sandwich dimer. The monomeric conformation binds and activates the receptor XCR1, while the dimer binds extracellular matrix glycosaminoglycans and has been associated with anti-human immunodeficiency virus (HIV) activity. Functional studies of WT-XCL1 are complex as both conformations are populated in solution. To overcome this limitation, we engineered a stabilized dimeric variant of XCL1 designated CC5. This variant features a neo-disulfide bond (A46C-A49C) that prevents structural interconversion by locking the chemokine into the β -sandwich dimeric conformation, as demonstrated by NMR structural analysis and hydrogen-deuterium exchange experiments. Functional studies analyzing glycosaminoglycan binding demonstrate that CC5 binds with high affinity to heparin. In addition, CC5 exhibits potent inhibition of HIV-1 activity in primary peripheral blood mononuclear cells (PBMCs), demonstrating the importance of the dimer in blocking viral infection. Conformational variants like CC5 are valuable tools for elucidating the biological relevance of the XCL1 native-state interconversion and will assist in future anti-viral and functional studies.

Introduction

Chemokines are a family of ~50 secreted signaling proteins that induce chemotactic cellular migration in a variety of physiological functions such as immune response, wound healing, and tissue maintenance ¹. Chemokines stimulate chemotaxis by activating specific G-protein coupled receptors (GPCRs) ¹, and adhering to extracellular matrix glycosaminoglycans (GAGs) ^{2,3}. Chemokines are grouped into subfamilies based on the configuration of two conserved disulfide bonds found within the N-terminus ⁴ (i.e. CXC, CC, CX₃C, C). All

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Accession Codes. Final structure calculations and models were deposited into the Biological Magnetic Resonance Data Bank (BMRB ID: 25693) and the RSCB Protein Data Bank (PDB: 2n54).

chemokines share a canonical tertiary fold comprised of a three-stranded β -sheet and C-terminal α -helix, and most of them self-associate to form dimers, higher-order oligomers⁵, or polymers⁶. It is generally understood that cell surface GPCR activation is associated with the chemokine monomer, while interactions with GAGs tend to promote oligomerization that aids in the formation of chemotactic gradients⁷.

Lymphotoxin (Ltn, XCL1), the defining member of the C-class subfamily, is unique in that it contains only one of the two conserved disulfide bonds typically associated with chemokines. Additionally, whereas most chemokines activate GPCRs and bind GAGs within a framework of the canonical fold, XCL1 exhibits reversible conformational heterogeneity and reversibly interconverts between two distinct structural states⁸. In one state, termed XCL1_{mon} (previously known as Ltn10, Table 1) the protein adopts a monomeric canonical fold that is capable of activating its cognate GPCR, XCR1⁹. However XCL1 can also access another unique conformational state, termed XCL1_{dim} (previously known as Ltn40, Table 1), comprised of a dimeric 4-stranded β -sheet structure capable of high affinity GAG interactions. This conformational switching involves unfolding of the protein and a complete restructuring of hydrogen bonding networks^{8,10}. The structural equilibrium can be shifted by changes in temperature and ionic strength, with high salt and low temperature favoring the monomeric chemokine fold (XCL1_{mon}), while low salt and high temperature favoring the alternative all- β sheet dimer (XCL1_{dim}). Under near-physiological conditions (37°C, 150 mM NaCl), the two conformational states are equally abundant and interconvert at a rate of $\sim 1 \text{ s}^{-1}$ ^{11,12}. Thus, XCL1 samples at least three distinct conformational states in solution: folded XCL1_{mon}, folded XCL1_{dim}, and unfolded.

Our goal is to resolve structure-activity relationships encoded within each conformational state. This includes the characteristic interactions with XCR1 and GAGs required for XCL1 to function as a chemoattractant *in vivo*. Like many chemokines and other cationic peptides XCL1 exhibits bactericidal activity¹³, and it was recently shown to be a broad-spectrum inhibitor of HIV-1¹⁴. Consequently, the scope of our XCL1 structure-function studies is expanding to include multiple types of antimicrobial activity. Although the relative populations of the two folded states in the wild-type protein can be manipulated to a degree, it is difficult to interpret *in vitro* or *in vivo* functional studies unless each conformation can be analyzed in isolation. In previous studies, we have used structure-guided protein engineering to generate conformationally selective XCL1 variants, summarized in Table 1. An XCL1 double mutant (V21C V59C; CC3) containing a second disulfide bond restricts the protein to the monomeric chemokine fold (XCL1_{mon}) and preserves full XCR1 agonist activity⁹. By selectively destabilizing the XCL1_{mon} chemokine conformation, the XCL1 W55D variant (W55D) enriches the XCL1_{dim} population and binds GAGs with high affinity but no longer activates XCR1⁸. Upon comparing the conformationally restricted XCL1 variants, Guzzo, et al. found that WT-XCL1 and W55D exhibited equally potent anti-HIV activity, while the CC3 variant was inactive¹⁴. While these results implicated XCL1_{dim} as the inhibitory state, a role for the unfolded state, which is equally accessible in the wild-type and W55D proteins but not CC3, could not be ruled out.

To enable XCL1 structure-function studies that fully isolate the XCL1_{mon}, XCL1_{dim}, and unfolded states, we engineered an XCL1 variant that is restricted to the dimeric 4-stranded

β -sheet fold (CC5, A36C-A49C) through introduction of a novel disulfide cross-link. NMR structural analysis indicates that the CC5 variant forms a more stable XCL1_{dim} than WT-XCL1 or W55D as determined by hydrogen/deuterium (H/D) exchange. CC5 binds heparin with a 15-fold higher affinity than CC3 and potently inhibits HIV-1, whereas an unfolded XCL1 variant is inactive. These results define a new structure-function relationship for the metamorphic native state of XCL1 and establish the CC5 locked XCL1_{dim} as a useful tool for future investigations.

RESULTS AND DISCUSSION

Protein Engineering

A new XCL1_{dim}-stabilizing variant was empirically designed by exploiting the unusual structural changes that occur upon XCL1_{mon}-XCL1_{dim} interconversion. Metamorphic rearrangement of the conserved chemokine fold (XCL1_{mon}) into the XCL1_{dim} involves a register shift and inversion of adjacent β -strands hydrogen bonding networks⁸. We reasoned that a disulfide joining adjacent β -strands would restrict structural interconversion, thus making the dimeric fold the only accessible conformation. For example, inspection of the high temperature XCL1_{dim} structure revealed two alanine residues (A36 and A49) separated by an α -carbon distance of ~ 2.5 Å (~ 6 Å separation between side chain β -carbons) and located on the adjacent β_2 and β_3 strands (Fig. 1A). Conversion to the XCL1_{mon} conformation repositions the A36 and A49 side chains on opposite faces of the β -sheet (Fig. 1B). This change in orientation and proximity revealed the potential for conformation-specific disulfide bond formation in the dimer. Cysteine mutations were introduced at these sites (i.e. A36C and A49C) and recombinant [U - ^{15}N , ^{13}C]-XCL1 CC5 was expressed in *E. coli*, refolded and purified to homogeneity for structural and functional characterization.

XCL1 CC5 is folded and non-metamorphic

A ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectrum for CC5 was acquired in 20 mM NaHPO₄, pH 6 at 40° C. The initial spectrum presented well-resolved cross peaks with uniform intensities accounting for 88% of expected backbone resonances and indicative of an ordered 3D structure. Comparison of CC5 to a WT-XCL1 HSQC spectrum acquired under identical conditions revealed numerous differences in NH backbone resonances that did not allow direct transfer of all chemical shift assignments (Fig. 1C, 40°C). The HSQC spectrum can be considered a diagnostic fingerprint of the protein backbone, sensitive to slight perturbations in electronic environments, and observation of chemical shift differences between CC5 and WT-XCL1 was not surprising considering that the mutations were designed to restrict conformational accessibility through introduction of a disulfide cross-link. Spectra were also collected and compared for both proteins at 25°C (0 M NaCl). Under these conditions, WT-XCL1 is known to be in equilibrium between both conformational states that are observable by NMR¹⁵. Interestingly at 25°C, CC5 showed no evidence of dual state behavior while WT-XCL1 presented clear indications of structural heterogeneity (highlighted boxes in Fig. 1C, 25°C).

To further assess the conformational stability of CC5, we compared its HSQC spectrum with XCL1 W55D over a range of temperatures. The W55D variant was designed to restrict

conformational equilibrium and select for XCL1_{dim} by destabilizing the hydrophobic core of XCL1_{mon}. Even though W55D does not access XCL1_{mon}, it is able to undergo an on-pathway unfolding event that is part of the WT conformational exchange. This is evident in the highlighted areas of the HSQC spectra shown in Fig. 1D. At higher temperatures the spectra for these variants show peak dispersion indicative of XCL1_{dim}, while spectra collected at lower temperatures exhibit weaker or absent signals that result from protein unfolding. This is consistent with measurements of urea-induced equilibrium unfolding of XCL1 W55D showing maximum thermostability at ~40 °C and significantly lower stability at 10 °C (RC Tyler and BF Volkman, *unpublished results*).

To confirm that the absence of dual-state behavior was caused by the introduction of a novel disulfide link between C36-C49, we analyzed 3D NMR spectra (i.e. ¹³C, ¹⁵N, ¹H) in order to determine ¹³Cα and ¹³Cβ chemical shift values, which indicate the oxidation state of cysteine residues in proteins. Specifically, if the Cβ chemical shift is less than 32.0 ppm or greater than 35.0 ppm, the redox state is assigned to reduced or oxidized, respectively ¹⁶. Analysis of NMR spectra indicated that all four cysteine residues of CC5 are in the oxidized state (¹³Cβ > 35 ppm), consistent with the presence of both the original XCL1 disulfide bond and the engineered C36-C49 disulfide (data not shown).

The assigned HSQC spectrum for CC5 is displayed in Supplemental Figure 1. All backbone and side chain chemical shifts for CC5 were verified or assigned from analysis of standard triple resonance experiments described in material and methods. The complete set of NMR data was deposited into the Biological Magnetic Resonance Bank (BMRB) under accession number 25693 and the RCSB Protein Data Bank (PDB: 2n54).

CC5 adopts the XCL1_{dim} fold

The structure of CC5 was determined by NMR spectroscopy at 40°C. All distance restraints used in the calculation were derived from ¹⁵N-edited and ¹³C-edited NOESY spectra. Resulting NOE cross peaks were assigned using an established protocol described in material and methods. Intermolecular NOEs between dimer subunits were identified from ¹³C-¹²C filtered NOESY spectra allowing determination of quaternary structure (Supplemental Figure 2). After final analysis a total of 1456 NOE distance restraints were determined and used in the calculation of the CC5 structure. A stereo view of the final ensemble of 20 conformers (PDB: 2n54) is shown in Fig. 2A, with a close up view of the engineered disulfide bond between C36-C49 shown in Fig. 2B. A summary of all experimental restraints and structural statistics generated for the ensemble is presented in Table 2.

The mutant structure displayed the expected 4 β-strands per subunit with the dimer displaying a two-fold axis of symmetry approximately perpendicular to the β-sheet. Comparison of the overall geometries comprising the mutant and WT-XCL1_{dim} folds revealed very similar regions of secondary structure (Fig. 2C) producing a Cα backbone alignment RMSD of 1.9 Å (residues 2-55). It is noted that the engineered C36-C49 disulfide bond linking the β₂/β₃ strands is buried within the hydrophobic core of the protein (Fig. 2B). This is consistent with the location of the analogous alanine methyl groups found within the

wild-type dimer fold and signifies that the engineered disulfide introduced minimal distortion of structural elements relative to the XCL1_{dim} structure.

H/D exchange NMR reveals increased amide backbone protection for CC5

The conformational dynamics of CC5 was probed by H/D exchange NMR. This experiment relies on chemical exchange between amide protons and NMR silent deuterons giving an indication of solvent accessibility of the protein backbone. Previous experiments with XCL1 are consistent with extremely rapid H/D exchange regardless of pH, temperature, or salt concentration indicating little protection of backbone amide protons within the dead time of the experiment (~ 5 min)¹¹. The lack of observable signal can be rationalized given the presence of metamorphic interconversion that likely requires global unfolding¹⁰. This unfolding allows for exposure of backbone amide hydrogen to solvent and results in the rapid exchange of ¹H-¹⁵N signals. The CC3 variant was designed to lock XCL1 into the XCL1_{mon} conformation with the addition of a disulfide that prevents unfolding and interconversion⁹. As expected, H/D exchange analysis of CC3 showed significant protection from amide exchange as evidenced by the persistence of ¹H signal ~ 20 minutes after deuterium addition (Fig. 3A). HSQC analysis comparing CC3 in both 10% D₂O and 100% D₂O solutions revealed 13 protected residues (Fig. 3B & C). Peak intensities for protected residues were measured from HSQCs collected over a time frame of 24 hours and used to calculate exchange rates (k_{ex}), protection factors (P), and free energy values (ΔG) (Supplemental Table 1). Residues that displayed the highest logP values (Fig. 3D) were localized in or near the hydrophobic pocket within the α -helix and the 1st and 2nd β -strands (Fig. 3E).

The W55D variant was originally designed to bias the XCL1 conformational equilibrium toward the XCL1_{dim} by disrupting the XCL1_{mon} hydrophobic core⁸. Attempts to measure H/D exchange rates for W55D after addition of 100% D₂O yielded no observable ¹H and HSQC signals after addition of 100% D₂O within the dead time of experiment (~ 5 min) (Fig. 3 F-H). This result suggests that, although the W55D mutation favors the XCL1_{dim} conformation (Fig. 3G & I), the XCL1_{dim}-unfolded transition leads to rapid solvent exchange as previously observed for WT-XCL1. In contrast, H/D exchange measurements on CC5 yielded significant ¹H protection (Fig. 3J) with HSQC analysis identifying 9 protected residues (C36, V37, I38, F39, I40, T41, K46, V47, and C48) upon 20 minutes of 100% D₂O exposure (Fig. 3K & L and Supplemental Table 1). These residues are localized to the internal strands (β_2 and β_3) of the dimer (Fig. 3M & N) and suggest that global unfolding of CC5 is significantly slowed relative to WT-XCL1 or W55D, by the engineered intramolecular disulfide bond.

Disulfide-locked XCL1 dimer binds heparin with high affinity

As the ability of chemokines to bind extracellular GAGs is largely dependent on surface exposed arginine, lysine, and histidine residues, the electrostatic surfaces comprising the structured regions of the XCL1_{dim} and the CC5 variant are displayed in Fig. 2D. Based on the structure it was apparent that the charge distribution of the disulfide mutant was similar to WT-XCL1_{dim}, suggesting that the mutant construct would also be capable of high affinity GAG binding. Reported heparin binding affinities (K_d) for XCL1 derived from surface

plasmon resonance (SPR) measurements range from about ~20 to 90 nM^{17, 18}. Therefore the ability of CC5 to bind to heparin was also investigated by SPR in order to determine binding affinity. In these experiments several concentrations of CC5 were applied to a heparin-coated SPR chip and resulting sensorgrams were analyzed. Initial measurements revealed robust responses of CC5 toward the heparin-coated SPR chip indicating significant interaction ($RU > 10$) (Fig. 4A). However, the response curves appeared to display multiphasic kinetic behavior indicative of complex binding, which precluded analysis using a simple bimolecular interaction model. This same phenomenon had been observed previously in similar SPR investigations involving XCL1/GAG interactions¹⁸. As a result, an apparent K_d value (K_d') was calculated from nonlinear fitting of the dose-dependent steady-state response of the interaction (R_{max}) (Fig. 4C). This equilibrium binding analysis yielded an affinity for heparin of approximately 59 nM, consistent with previous measurement of XCL1-GAG binding and confirming that CC5 preserves the high-affinity GAG binding conformation. For comparison with a disulfide stabilized version of the XCL1 chemokine fold, we measured heparin binding to the CC3 monomer under identical solution conditions used to investigate CC5 (Fig. 4B). Despite the fact that CC3 retains the same overall charge and number of Arg, Lys, and His residues as WT-XCL1, SPR measurements yielded a $K_d' \sim 1 \mu M$, approximately 15 times weaker than dimeric CC5 (Fig. 4C).

We also noted a disparity in R_{max} at equivalent concentrations of CC5 and CC3. The recent work of Salanga and co-workers investigated the role of chemokine oligomerization in GAG binding and observed a similar reduction in SPR response units when a non-oligomerizing variant of CCL2 (P8A) was applied to a heparin surface¹⁹. This reduced SPR signal associated with P8A-CCL2 was accompanied by a 10-fold reduction in heparin binding relative to WT-CCL2, illustrating the role of chemokine self-association in modulating GAG binding affinity¹⁹. We conclude that the dramatically lower R_{max} of CC3 derives from the lack of XCL1 self-association, whereas CC5 can form a more extensive chemokine-GAG interface and may interact with multiple heparin molecules. Taken together, SPR analysis of heparin binding indicates that the alternative XCL1 dimer is the dominant GAG binding structure and that oligomerization of the chemokine is critical for high-affinity XCL1-GAG interactions.

CC5 is a potent HIV-1 inhibitor

In a recent publication Guzzo, et al. demonstrated that both WT XCL1 and the W55D dimer were potent inhibitors of HIV-1 infection in human peripheral blood mononuclear cells (PBMCs) and in an engineered target cell line¹⁴. Analysis of the locked monomer (CC3) revealed that the canonical chemokine fold of XCL1 displayed no anti-HIV inhibition¹⁴. However, due to the relative instability of the W55D variant, it remained to be determined whether the HIV-1 inhibition is specifically associated with the dimeric conformation or results from access to an unfolded state. To resolve this question, HIV-1 inhibition assays were performed by comparing the effects of CC5 with those of WT, W55D, CC3, and an unfolded variant (C11A-C48; CC0) of XCL1. The CCR5 chemokine ligand CCL5 and the CXCR4 ligand CXCL12 were included as positive and negative controls, respectively, for competitive inhibition of chemokine receptor binding by the BaL (CCR5-tropic) HIV-1 isolate. CC5 as well as the variants that have access to the XCL1_{dim} exhibited HIV-1

inhibition, indicating that anti-viral activity is associated with the dimeric all- β structure of XCL1 (Fig. 4D). Both CC0 and CC3 were ineffective at HIV-1 inhibition. These results show that the anti-HIV activity is associated exclusively with the alternative dimer conformation and not with the canonical chemokine monomer or the unfolded state of XCL1.

Discussion

XCL1 defines a new category of metamorphic proteins, polypeptides that interconvert between unrelated structures in the native state^{8, 20}. Since these structures carry out distinct functions, protein metamorphosis represents a new and potentially widespread regulatory mechanism. However, because conventional structural methods tend to exclude or obscure metamorphic proteins, the sequence characteristics that define them remain to be uncovered. In parallel with our efforts to discover the origin of metamorphic XCL1 folding, we are systematically defining structure-function relationships for each accessible conformation.

Because XCL1 unfolds and interconverts readily between its metamorphic states, stabilized variants for the monomer and dimer are needed for functional studies of each conformation. XCL1 CC3 preserves XCR1 binding and activation, while W55D has been used to represent the XCL1_{dim} state and study XCL1-GAG interactions and anti-HIV activity. W55D is distinct from the disulfide-locked CC3 variant because it accesses an unfolded intermediate state that is more analogous to WT-XCL1 interconversion. In this work, we developed a new disulfide-locked variant (CC5, A36C-A49C) that adopts the XCL1 dimer structure with a longer lifetime than the W55D single mutant, as gauged by H/D exchange, and retains high affinity GAG binding. At lower temperatures, both W55D and CC5 display reduced stability (Fig. 1D), suggesting that these variants retain essential thermodynamic characteristics of the wild-type XCL1_{dim} conformation. With the advent of CC5, we now have a toolkit of variants designed to isolate different subsets of the XCL1 conformational states sampled by the native metamorphic protein (Table 1).

Recent studies have shown that XCL1 is important for facilitating inflammatory interactions between XCR1⁺ dendritic cells and CD8⁺ positive T cells as well as mediating a homeostatic role in the maintenance of self-tolerance through the development of T regulatory (Treg) cells in the thymus²¹. While the specific role remains to be determined, we speculate that metamorphic XCL1 interconversion is necessary for its roles in antigen presentation and immune system development. Conformationally restricted XCL1 variants, like CC5, are valuable tools for elucidating the biological relevance of the XCL1 native-state interconversion.

Besides the chemoattractant and GAG-binding functions of XCL1, we are beginning to understand the role of metamorphic equilibrium in the context of viral infection. XCL1 variants that access the dimeric β -sheet fold (XCL1_{dim}) are potent inhibitors of HIV-1 infection, while the canonical chemokine fold (XCL1_{mon}) is ineffective. XCL1 is able to block HIV-1 infection via an unconventional mechanism which is not mediated by engagement of viral co-receptors²², but rather by direct binding to the external viral envelope glycoprotein, gp120¹⁴. Taken together, this work further established unique roles for the different structural states of XCL1 in anti-HIV activity. In addition to HIV, other

viruses such as herpesviruses have been shown to disrupt/exploit XCL1-XCR1 signaling through the production of XCL1 and XCR1 mimics^{18, 23, 24}. It is reasonable to speculate that the conformational states of XCL1 may be implicated in other pathogenic pathways. Perhaps microbial evolutionary pressures have selected for the unique conformational equilibrium of XCL1 and access to the XCL1_{dim} state to combat invading microbes. Our future goal is to use CC5 and other XCL1 variants to better understand XCL1 biology in the context of its direct antimicrobial activity and XCR1-mediated immune functions.

METHODS

Mutagenesis, protein expression and purification

Site directed mutagenesis was performed on a pQE30 vector containing WT-XCL1 using pairs of complementary primers and the QuickChange kit to incorporate cysteine to alanine mutations at positions 36 and 49 relative to the protein primary sequence. The resulting plasmid was transformed into SG13009 (pREP4) and grown on M9 minimal medium containing ¹⁵N-ammonium chloride and ¹³C-glucose as the sole nitrogen and carbon sources. Cultures were grown to OD ~1 and protein expression induced by addition of 1mM isopropyl β-D-1-thiogalactopyranoside at 37°C. Subsequent purification and refolding of CC5 as well as the other XCL1 variants was accomplished following established methods that have been previously described¹⁷.

NMR spectroscopy

NMR experiments were performed at 40°C on a Bruker DRX600 equipped with a triple-resonance cryogenic probe. Samples were prepared in 20 mM NaHPO₄, pH 6.0 at ~1 mM protein concentration. Complete resonance assignments were derived from the following experiments: HNCA, HNCO, HNCACB, HNCOCA, HNCACO, CCONH, HBHACONH, HCCONH, HCCH-TOCSY. Data processing and analysis were performed using NMRPipe²⁵ and XEASY software packages²⁶.

Structure Calculation

Structures of CC5 were calculated using NOE distance restraints obtained from 3D ¹⁵N-edited NOESY-HSQC, ¹³C(aliphatic)-edited NOESY-HSQC, ¹³C(aromatic)-edited NOESY-HSQC, and ¹³C-¹²C-filtered NOESY-HSQC (Supplemental Figure 2). Additional backbone dihedral angle restraints (φ and ψ angles) were derived from ¹Hα, ¹³Cα, ¹³Cβ, ¹³CO, ¹⁵N chemical shift data using TALOS²⁷. Structures were generated in an automated manner using the NOEASSIGN module of the torsion angle dynamics program CYANA 2.1²⁸. This method produced an ensemble of high precision structures that required minimal manual refinement. The 20 CYANA conformers with lowest target function were further refined by a molecular dynamics protocol in explicit solvent²⁹ using XPLOR-NIH³⁰.

HD Exchange

Purified, recombinant proteins (XCL1 W55D, CC5, and CC3) were analyzed for structural stability by hydrogen/deuterium (H/D) exchange NMR. Lyophilized protein were initially suspended in 20 mM NaHPO₄ (pH 6.0) with 10 % D₂O to a final concentration of 1 mM. 1D ¹H and 2D ¹H-¹⁵N HSQC were collected at 25 °C on a Bruker Avance 500 MHz

spectrometer to verify the protein structure. These samples were dried on a speed vac in the buffer solution and resuspended in 100 % D₂O. A series of 2D ¹H-¹⁵N HSQCs were collected over time (~24 hours) at 25 °C to monitor the persistence of amide hydrogen signals. Peaks that demonstrated prolonged protection from deuterium exchange were identified based on previous NMR assignments and analyzed by non-linear fitting of an exponential decay function to calculate H/D exchange rates (k_{ex}) using ProFit software (QuantumSoft). Protection factors (Log(P)) and free energy values (ΔG) were calculated using FBMME HD exchange spreadsheets (<http://hx2.med.upenn.edu>).

Surface Plasmon Resonance

Analysis of XCL1/heparin interactions was done using a BIAcore 3000 instrument equipped with a C1 sensor chip. The C1 sensor chip was prepared by activation with a 1:1 mixture of NHS and EDC (300 μ L at 20 μ L/min), followed by immobilization of Neutravidin (Invitrogen) at 20 μ L/min in 10 mM NaOAc, pH 6, then deactivation of excess groups with ethanolamine and finally washing of the surface with 10 mM NaOAc, pH 5.5 buffer prior to heparin addition. Biotinylated heparin (Sigma) was applied to the activated C1 chip to saturation based on response units. For analysis, varying concentrations of protein (50, 100, 200, 250, 400, 500, 750, and 1000 nM) was applied to the chip for 5 min at 40 μ L/min in SPR running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Tween-20, pH 7.4), followed by 5 min of dissociation. Surfaces were regenerated after each injection using 0.1 M glycine, 1 M NaCl and 0.1% Tween-20, pH 9.5 (31). All K_d values were determined from steady state analysis using an average of maximum response value generated for each protein concentration, and fit with the BIAevaluation software (BIAcore) using a 1:1 association model.

HIV-1 infection assays

The BaL (R5-tropic) laboratory strain of HIV-1 was used to infect human PBMCs as previously described ¹⁴. Briefly, PBMCs were isolated from individuals and activated with PHA and IL-2 for 72 hrs. The cells were resuspended in RPMI + 10% FBS + 20 U/mL of IL-2 and plated at 2×10^5 cells/well in a round-bottom 96-well plate in duplicate. HIV-1 infection was initiated by addition of 100 pg of p24 Gag antigen to each well. Infected cells were incubated with and without XCL1 proteins in concentrations ranging from 0.12 – 1 μ M. HIV replication was assayed by measuring the extracellular release of p24 Gag protein in supernatants harvested from cell cultures 4 days post-infection. The Alpha (Amplified Luminescent Proximity Homogenous Assay) technology immunoassay (AlphaLISA HIV p24 Research Immunoassay Kit, PerkinElmer) was used to measure p24 levels. Data were normalized to control cultures and values are represented as mean and standard deviation of three replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CC5 is engineered to be non-metamorphic. a) Representation of WT-XCL1_{dim} structure (PDB-2JP1) illustrating the rational design of the CC5 variant. Mutation of A36C-A49C was chosen due to their proximity within the XCL1_{dim}. b) Representation of the XCL1_{mon} structure (PDB-1J8I) illustrating that A36 and A49 are positioned on opposing surfaces of β -strands. Mutation of A36C and A49C is not expected to not be conducive to disulfide bond formation in this state. c) Comparison of WT-XCL1 (green) and CC5 (black) HSQC spectra acquired at 25°C and 40°C in 20 mM NaHPO₄, pH 6. The dashed boxes in the 25°C

spectrum denote areas in WT-XCL1 that reveal evidence of conformational heterogeneity that is absent for CC5. d) HSQCs of XCL1 W55D and CC5 collected in 20 mM NaHPO₄ (pH 6) at the indicated temperatures. Dashed boxes highlight residues that are indicative of the XCL1_{dim} structure.

Figure 2.

XCL1 CC5 represents the unique β -sandwich dimer. a) Stereo view of the twenty CC5 conformers generated from structure calculation. b) Close-up view of the disulfide bonds within protein subunit. c) Overlay generated from C α -trace (residues 2-55) of the lowest energy structures of CC5 mutant (green) and WT-XCL1_{dim} (cyan). A side view of the overlaid structures is also shown (90° rotation around the vertical axis). d) Comparison of electrostatic surfaces; (blue) positive charge, (red) negative charge, (white) neutral.

Figure 3.

H/D exchange analysis demonstrates the stability of CC5. Protein samples were diluted to a final concentration of ~ 1 mM in 20 mM NaHPO_4 (pH 6.0) and D_2O was added as indicated.

a) ^1H -NMR spectrum of XCL1 CC3 (locked monomer) in 10% D_2O (black) and ~20 min after 100% D_2O was added (cyan). b) HSQC of CC3 in 10% D_2O . c) HSQC of CC3 ~20 min after 100% D_2O was added. d) Log(P) plot of protected residues in CC3. Protection factors were calculated from observable peak intensities and displayed as Log (P). e) Protected residues (cyan) mapped onto the structure of CC3 (PDB-2HDM). The disulfide

bonds are represented as yellow sticks. f) ^1H -NMR spectrum of W55D (preferential dimer) in 10% D_2O (black) and ~20 min after 100% D_2O was added (cyan). g) HSQC of W55D in 10% D_2O . h) HSQC of W55D ~20 min after 100% D_2O was added. i) Structure of the XCL1_{dim} conformation of XCL1 WT (PDB-2JP1). No protected residues were measured for W55D. j) ^1H -NMR spectrum of CC5 in 10% D_2O (black) and ~20 min after 100% D_2O was added (cyan). k) HSQC of CC5 in 10% D_2O . l) HSQC of CC5 ~20 min after 100% D_2O was added. m) Log(P) plot of protected residues in CC5. n) Protected residues (cyan) mapped onto the structure of CC5 (PDB: 2n54). See also Supplemental Table 1.

Figure 4.

CC5 binds with high affinity to heparin and potently inhibits HIV-1 infection. SPR sensorgrams comparing CC5 a), and CC3 b) at varying protein concentrations (shown on curves) applied over heparin chip. For CC3, protein concentrations below 200 nM were removed for clarity. Identical scaling was used in both sensorgrams. c) Heparin binding curves generated from steady-state analysis used to determine apparent binding affinity (K_D). R_{eq} represents an average of max response at each protein concentration. d) Comparison of HIV-1 inhibition in human PBMCs by WT-XCL1, W55D, CC3, CC5, and an

unfolded XCL1 variant (CC0). Activated PBMCs were treated with XCL1 proteins in concentrations ranging from 0.12 to 1 μ M and then exposed to the BaL strain of HIV-1 (CCR5 tropic). Infection was quantified by measuring the release of p24 Gag protein in supernatants harvested from the cultures four days post-infection. Data were normalized to untreated controls and presented as mean values $\pm$ standard deviation of replicate wells. CCL5 and CXCL12 are used as positive and negative controls for CCR5-tropic and CXCR4-tropic HIV-1, respectively.

Table 1

Nomenclature of XCL1 variants

	Nomenclature		Structure			Function			
	Name	Previous designation	Mutations	Fold	Oligomeric state	XCRI	GAG	HIV	Ref
Native states	XCL1 _{mon}	Ltn10	–	chemokine	monomer	Y	N	N	
	XCL1 _{dim}	Ltn40	–	β-sandwich	dimer	N	Y	Y	<i>a</i>
	XCL1 _{unf}	–	–	unfolded	Monomer	N	N	N	
Conformationally-restricted variants	XCL1	Lymphotoctin; ATAC	–	metamorphic	monomer-dimer equil.	Y	Y	Y	<i>a, b, c</i>
	W55D	W55D	W55D	XCL1 _{dim}	dimer	N	Y	Y	<i>d</i>
	CC0	C11/48A	C11A C48A	unfolded	n.d.	N	N	N	
	CC1	CC1	T10C and -AC- inserted between G32 and S33	XCL1 _{mon}	monomer	n.d.	n.d.	n.d.	
	CC2	CC2	P20C V59C	n.d.	n.d.	n.d.	n.d.	n.d.	<i>e</i>
	CC3	CC3	V21C V59C	XCL1 _{mon}	monomer	Y	N	N	<i>a, e</i>
	CC4	–	T26C F39C	n.d.	n.d.	n.d.	n.d.	n.d.	
	CC5	A36C A49C	A36C A49C	XCL1 _{dim}	dimer	N	Y	Y	

*a*₁₄*b*₃₁*c*₃₂*d*₈*e*₉

Table 2

Structural statistics for the XCL1 CC5 ensemble

Experimental constraints	PDB code: 2n54	
Distance constraints		
Intermolecular		90
Long		658
Medium [1<(i-j) 5]		186
Sequential [(i-j)=1]		142
Intra-residue [i=j]		380
Total		1456
Dihedral angle constraints (ϕ and ψ)		108
Average atomic R.M.S.D. to the mean structure (Å)		
Dimer – Residues 10-51,210-251		
Backbone (C ^α , C′, N, O)		0.66 ± 0.08
Heavy atoms		1.22± 0.11
Monomer A – Residuaes 10-51		
Backbone (C ^α , C′, N, O)		0.56 ± 0.09
Heavy atoms		1.16 ± 0.10
Monomer B – Residues 210-251		
Backbone (C ^α , C′, N, O)		0.56 ± 0.10
Heavy atoms		1.15 ± 0.14
Deviations from idealized covalent geometry		
Bond lengths	RMSD (Å)	0.018
Torsion angle violations	RMSD (°)	1.5
WHATCHECK quality indicators		
Z-score		−3.20 ± 0.24
RMS Z-score		
Bond lengths		0.86 ± 0.02
Bond angles		0.73 ± 0.02
Bumps		0 ± 0
Lennard-Jones energy ^a (kJ mol ^{−1})		−3,664 ± 128
Constraint violations		
NOE distance	Number > 0.5 Å	0 ± 0
NOE distance	RMSD (Å)	0.022 ± 0.001

Experimental constraints		PDB code: 2n54
Torsion angle violations	Number > 5 °	0 ± 0
Torsion angle violations	RMSD (°)	0.701 ± 0.103
Ramachandran statistics (% of all residues)		
Most favored		72.3 ± 3.8
Additionally allowed		23.4 ± 3.5
Generously allowed		1.9 ± 1.1
Disallowed		2.4 ± 2.1

^aNonbonded energy was calculated in XPLOR-NIH.