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Structural Determinants for the Selective Anti-HIV-1 Activity of the All-β Alternative Conformer of XCL1

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ABSTRACT

HIV-1 replication is regulated *in vivo* by a complex network of cytokines and chemokines. XCL1/lymphotactin, a unique metamorphic chemokine, was recently identified as a broad-spectrum endogenous HIV-1 inhibitor that blocks viral entry via direct interaction with the gp120 envelope glycoprotein. HIV-1 inhibition by XCL1 requires access to the alternative all-β conformation, which interacts with glycosaminoglycans (GAGs) but not with the specific XCL1 receptor, XCR1. To investigate the structural determinants of the HIV-inhibitory function of XCL1, we performed a detailed structure-function analysis of a stabilized all-β variant, XCL1 W55D. Individual alanine substitutions of two basic residues within the 40s' loop, K42 and R43, abrogated the ability of XCL1 to bind to the viral envelope and block HIV-1 infection; moreover, a loss of HIV-inhibitory function, albeit less marked, was seen upon individual mutation of three additional basic residues: R18, R35, and K46. In contrast, mutation of K42 to arginine did not cause any loss of function, suggesting that the interaction with gp120 is primarily electrostatic in nature. Strikingly, four of these five residues cluster to form a large (~350 Å^2) positively charged surface in the all-β XCL1 conformation, whereas they are dissociated in the classic chemokine fold, which is inactive against HIV-1, providing a structural basis for the selective antiviral activity of the alternatively folded XCL1. Furthermore, we observed that changes to the N-terminal domain, which is proximal to the cluster of putative HIV-1 gp120-interacting residues, also affect the antiviral activity of XCL1. Interestingly, the complement of residues involved in HIV-1 blockade is partially overlapping, but distinct from those involved in the GAG-binding function of XCL1. These data identify key structural determinants of anti-HIV activity in XCL1, providing new templates for the development of HIV-1 entry inhibitors.

IMPORTANCE

The host immune system controls HIV-1 infection through a wide array of inhibitory responses, including the induction of cytotoxic effector cells and the secretion of noncytolytic soluble antiviral factors such as cytokines and chemokines. We recently identified XCL1/lymphotactin, a chemokine primarily produced by CD8$^+$ T cells, as a novel endogenous factor with broad anti-HIV activity. Strikingly, only one of the two conformations that XCL1 can adopt in solution, the alternative all-β fold, mediates antiviral activity. At variance with the classic HIV-inhibitory chemokines such as CCL5/RANTES, XCL1 acts via direct interaction with the external viral envelope glycoprotein, gp120. Here, we identify the interactive surface of XCL1 that is implicated in binding to the HIV-1 envelope and HIV-1 inhibition, providing a structural basis to explain why only the all-β XCL1 conformer is effective against HIV-1. Our findings may be useful in guiding the rational design of new inhibitors of HIV-1 entry.

The natural history of HIV-1 infection is highly heterogeneous in different individuals, ranging from a steady asymptomatic condition to a rapid disease evolution (1). A major determinant of the pace of disease progression is the level of HIV-1 replication, which is regulated *in vivo* by an intricate network of bioactive molecules, including both soluble immune mediators and cell surface receptors. We recently reported that the C-chemokine XCL1/lymphotactin is a conformation-dependent broad-spectrum inhibitor of HIV-1 infection, which acts at the level of viral entry via an unconventional mechanism mediated by direct interaction with the external envelope glycoprotein, gp120 (2). XCL1 is a peculiar metamorphic chemokine that interconverts in solution between two distinct conformations: a monomeric chemokine-like fold (Ltn10), which binds and activates the cognate receptor, XCR1, and an alternatively folded, all-β conformation (Ltn40), which has a marked propensity to self-associate as a head-to-tail dimer and does not bind/activate XCR1 but rather interacts with cell surface glycosaminoglycans (GAGs) with high affinity (3, 4). It has been postulated that XCL1 requires access to these distinct conformations to perform unique but complementary functions, whereby the GAG-binding conformation facilitates the formation of chemokine gradients required to drive the migration of lymphocytes, and the chemokine-like conformation engages the specific XCR1 receptor to initiate intracellular signaling and biological responses (5). Using XCL1 variants stabilized in each of the two alternative conformations, we found that inhibition of HIV-1 requires access to the GAG-binding, alternative conformation, while the XCR1-binding (classic, chemokine-like) fold lacks anti-

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viral function. However, by enzymatic removal of GAGs on HIV-1 target cells, we demonstrated that interaction with cell surface GAGs is not required for the antiviral activity of XCL1, in agreement with the evidence that HIV-1 inhibition is mediated by direct interaction of the chemokine with gp120 (2). Altogether, these observations point to a role of electrostatic interactions, potentially related to those involved in GAG binding, in XCL1 interaction with gp120 (6–8).

Initial work investigating the GAG recognition determinants in XCL1 by alanine scanning mutagenesis identified two mutations, R23A and R43A, which resulted in the largest reduction in heparin-binding affinity relative to that of wild-type XCL1 (3). More recently, an extensive analysis of GAG-binding activity was performed in the context of a stabilized alternatively folded XCL1 conformation (Ltn40, here designated W55D), leading to the identification of a broader panel of residues involved in GAG binding (J. C. Fox et al., unpublished data). In the present study, we investigated the structural determinants of the anti-HIV-1 activity of XCL1, with specific emphasis on the role of basic amino acids and their relation to GAG-binding determinants. Our mutagenesis analysis confirmed the primarily electrostatic nature of the interaction of XCL1 with gp120. We mapped the antiviral function to a group of positively charged residues clustered to form a large basic surface which is present only in the alternatively folded, all-β XCL1 conformation but not in the classic chemokine fold, thereby providing a structural basis for the selective antiviral activity of the alternative conformer. The complement of residues responsible for HIV-1 blockade is partially overlapping but clearly distinct from the GAG-interactive residues, indicating that the antiviral and GAG-binding functions of XCL1 can be uncoupled.

MATERIALS AND METHODS

XCL1 mutants. XCL1 W55D and alanine mutants were cloned and produced by two of the authors (J. C. Fox and B. F. Volkman) at the Medical College of Wisconsin, Milwaukee, WI, as previously described (4). Alanine mutants in all basic residues of the structured, all-β core in the W55D XCL1 background were assayed, with the exception of R57A, since 2D-HSQC analysis indicated that this mutation compromised the stability of the protein folding. An additional K42R mutant was created to analyze the role of electrostatic interactions in XCL1 binding to HIV-1 gp120. Nuclear magnetic resonance (NMR) spectra confirmed that all of the mutants included in this study retained the correct all-β, alternatively folded XCL1 conformation (Fox et al., unpublished). Molar values were calculated based on the molecular weight of the monomeric chemokines. XCL1 variants with altered N termini were obtained from R&D Systems (Met-XCL1) and Peprotech (XCL1 2-93, with valine 1 removed).

HIV-1 isolates and infection assays. The replication-competent HIV-1 isolates used in the present study were two laboratory strains, IIIB (X4-tropic) and BaL (R5-tropic), produced and passaged exclusively in primary human peripheral blood mononuclear cells (PBMCs). PBMCs from healthy donors were activated with phytohemagglutinin (PHA; Sigma, St. Louis, MO) and recombinant human interleukin-2 (IL-2; Roche Applied Science, Mannheim, Germany) in complete RPMI medium (Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum (FBS; HyClone, Thermo Scientific, Waltham, MA), glutamine at 2 mM, streptomycin at 50 μg/ml, and penicillin at 100 U/ml for 72 h prior to HIV-1 infection. Standard infection assays were performed in TZM-bl cells, as previously validated and described for the routine screening of infection inhibition (9, 10). TZM-bl cells (NIH AIDS Research and Reference Reagent Program, Germantown, MD) were maintained in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% FBS. Briefly, infection assays were performed in flat-bottom 96-well plates, with viral input standardized for 200,000 relative light units per well. TZM-bl cells were seeded at 10^4 cells per well, followed by 48 h of incubation at 37°C for firefly luciferase reporter gene expression. Luciferase quantitation was performed using One-Step Bright-Glo (Promega) lysis and substrate addition, according to the manufacturer's instructions. For infection assays in primary cells, acute cell-free HIV-1 infection was performed by addition of the viral stocks (50 to 100 pg of p24 Gag antigen per well) to duplicate cultures of activated normal human PBMCs (PHA + IL-2 for 72 h) in round-bottom 96-well plates seeded at 2×10^5 cells per well in RPMI + 10% FBS + 20 U of IL-2/ml. Infected cells were cultured in the presence or absence of XCL1 mutant at doses ranging from 0.06 to 1 μM. HIV-1 replication was assessed by measuring the extracellular release of p24 Gag protein in cell-free culture supernatants taken daily between days 3 and 7 postinfection using a highly sensitive Alpha (amplified luminescent proximity homogeneous assay) technology immunoassay (AlphaLISA HIV p24 research immunoassay kit; Perkin-Elmer, Waltham, MA). On day 7 of infection, the cells were thoroughly resuspended and harvested for absolute viable cell counting by timed flow cytometry using appropriate forward and side scatter gating on live lymphocytes. Cell viability was determined by normalization of the total live-gated cell counts in XCL1-treated wells to the number of cells recovered from control wells (untreated with XCL1).

HIV-1 virion capture assay. The virion capture assay was performed as previously described (11). Briefly, immunomagnetic beads (4×10^4 per tube) covalently linked to a polyclonal antiserum to rabbit IgG (Invitrogen) were incubated with a polyclonal rabbit IgG antibody to human XCL1 (Peprotech), washed with phosphate-buffered saline (PBS) containing 0.02% (wt/vol) bovine casein, and then loaded with XCL1 mutants (2.5 μg per reaction). After the removal of unbound XCL1 by repeated PBS-casein washes, chemokine-armed beads were incubated with 0.2 ml of the viral stock (HIV-1 BaL [R5] or IIIB [X4]; 18 or 10 ng of p24 Gag protein/test). After incubation with virus overnight at 4°C, the beads were washed to remove unbound virus particles and treated with 0.5% Triton X-100 to lyse the captured virions. The amount of captured p24 Gag protein was quantified by AlphaLISA.

RESULTS

Positively charged amino acids mediate the antiviral activity of XCL1. Binding to GAGs is the main function of the alternative, all-β XCL1 conformation, which has been selectively associated with HIV-1 inhibition (2). To investigate the relationship between the antiviral and GAG-binding activities of XCL1, we generated a panel of mutants bearing individual alanine substitution of all basic amino acids in the structured core of XCL1 (amino acids 1 to 54), as well as three residues within the C-terminal tail (12). Since mutation of some basic residues was previously shown to shift the conformational equilibrium of XCL1 toward the monomeric chemokine conformation (13), which lacks HIV-inhibitory activity (2), mutagenesis was performed on the stabilized XCL1 variant W55D, in order to ensure that the functional impact of each substitution could be interpreted in the context of the all-β dimeric structure (2). All of the mutants were tested for antiviral activity against a reference CCR5-tropic (R5) HIV-1 isolate (BaL) in susceptible target cells (TZM-bl). As seen in Fig. 1A, individual alanine substitutions of two basic residues in the 40's loop, K42 and K43, were sufficient to completely abrogate the HIV-1 inhibitory activity of XCL1 W55D. A loss of inhibitory function, albeit less marked, was also observed with three additional mutants, R18A, R35A, and K46A. Of note, mutation of K9 and K23, which have been identified as key residues for GAG interaction (3; Fox et al., unpublished), did not affect the antiviral activity of XCL1. Similar results were observed with a CXCR4-tropic (X4) virus, IIIB, as well as using primary PBMC as target cells (data not shown), dose-response curves in PBMC for the two most effective mutations,

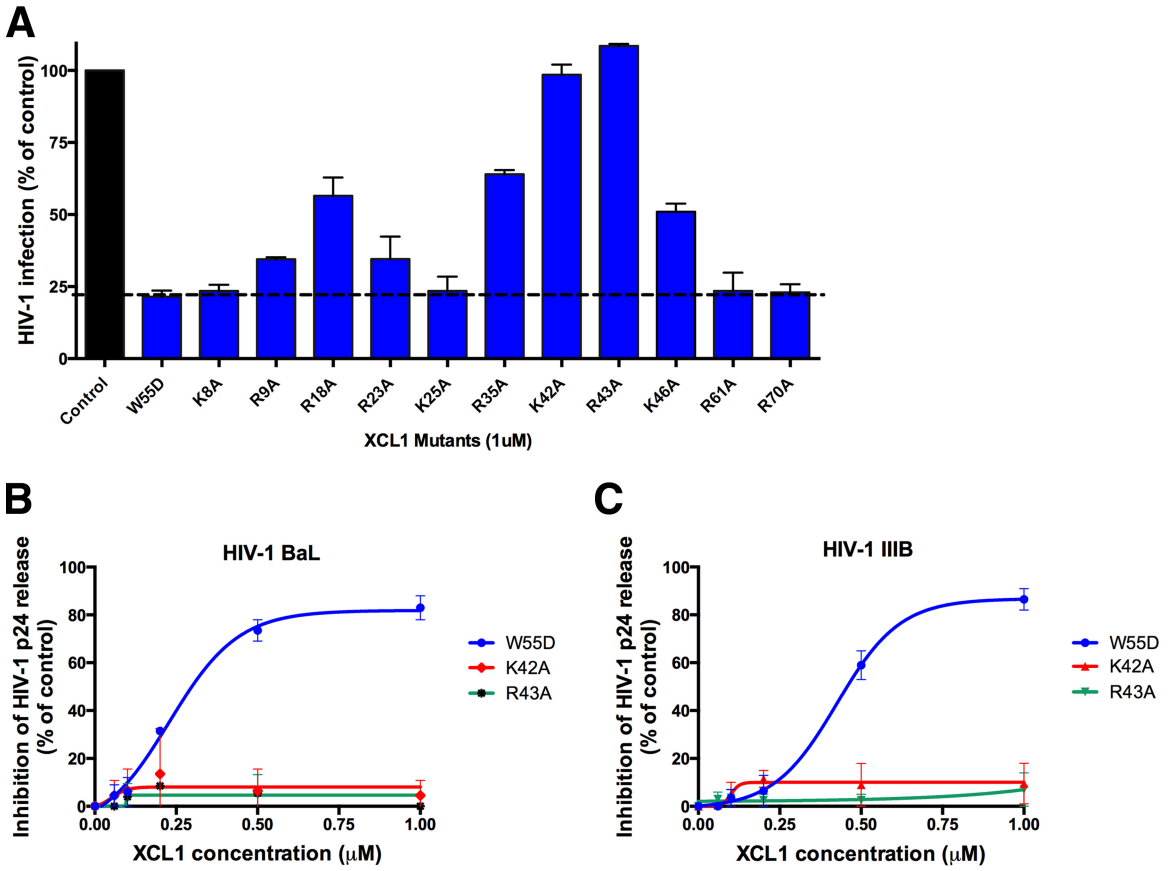

FIG 1 Loss of antiviral function by alanine substitution of basic residues in XCL1. (A) Effect of alanine substitution of basic residues in XCL1 W55D on HIV-1 infection in TZM-bl cells. HIV-1 inhibition assays were performed using 1 μM XCL1 variants against the prototypic R5 strain, HIV-1 BaL. The level of HIV-1 replication in the presence of XCL1 mutants, relative to control cultures not treated with XCL1, was measured by luciferase activity at 48 h postinfection and represents the mean (± the standard deviation [SD]) of replicate experiments. The dotted line represents the level of inhibition observed with the unmutated all-β XCL1 variant, W55D. (B and C) Dose-dependent inhibition of prototypic R5 (BaL) (B) and X4 (IIIB) (C) HIV-1 strains in cultures of activated primary human PBMCs. Virus replication was quantified by the amount of p24 Gag antigen in the culture supernatants via AlphaLISA immunoassay. The data were normalized to the amount of viral replication observed in control cultures (not treated with XCL1). The data represent mean values (± the SD) from replicate wells, representative of at least three independent experiments performed on PBMCs from different donors.

K42A and R43A, against both an R5 and an X4 virus are shown in Fig. 1B and C, respectively. These data demonstrated that specific basic residues play a key role in the anti-HIV activity of XCL1, suggesting that electrostatic interactions with gp120 mediate the inhibitory function of this chemokine.

Basic residues within XCL1 mediate direct interaction with the HIV-1 envelope. Virion capture assays were performed to assess the impact of alanine substitutions on the ability of XCL1 to directly bind mature HIV-1 virions, which was previously established as the principal mechanism of HIV-1 inhibition by this chemokine (2). As a preliminary quality control test, beads armed with the various XCL1 mutants were stained with polyclonal anti-XCL1 antibodies and analyzed by flow cytometry to verify whether beads were armed with comparable amounts of each XCL1 mutant (data not shown). As observed in infection assays, the same two residues from the 40s' loop, K42 and R43, were found to play the most pivotal role in mediating direct interaction with native HIV-1 virions, as indicated by complete abrogation of virion capture upon their alanine substitution (Fig. 2). Moreover, a drastic reduction in virion capture was also seen upon mutation of K46, whereas R18 and R35 seemed to play a lesser role in the

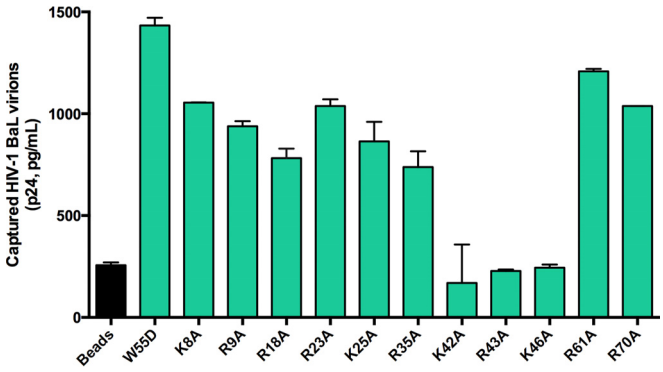

FIG 2 Effect of basic residue substitutions on the ability of XCL1 to capture HIV-1 virions. The virion-capture assay was performed using immunomagnetic beads armed with different XCL1 mutants (as indicated) as molecular baits to capture HIV-1 BaL virions from live viral stocks produced by primary human PBMCs. The black bar represents the background capture level observed with control, unarmed beads. All bars represent the average p24 captured in replicate experiments (± the SD).

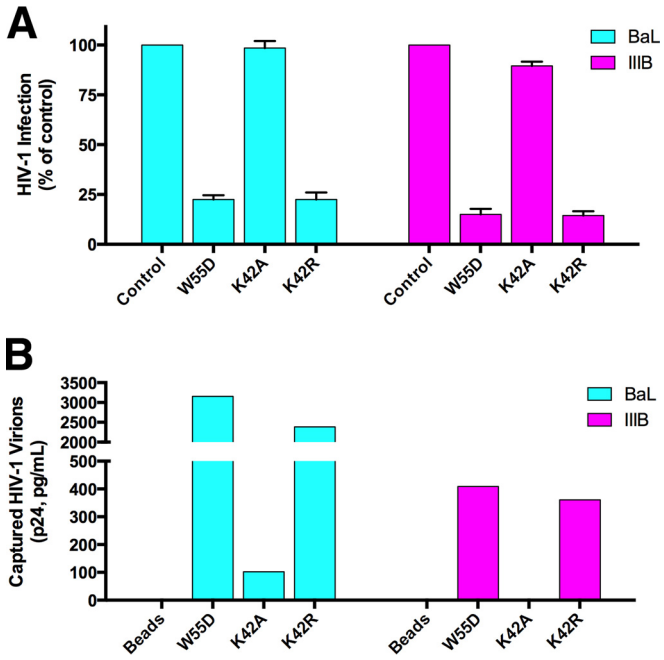

FIG 3 Role of electrostatic interactions in XCL1 anti-HIV activity. (A) TZM-bl neutralization assays were performed with 1 μM concentrations of XCL1 variants against the R5-tropic BaL virus (shown in turquoise) or the X4-tropic IIIB virus (magenta). Bars indicate the level of HIV-1 replication in the presence of XCL1 mutants (W55D, K42A, and K42R) compared to infection levels in control cultures (no XCL1 treatment). Replication was measured by luciferase readout 48 h postinfection; bars represent the average (± the SD) of replicate experiments. (B) Virion capture assays were performed using immunomagnetic beads armed with XCL1 mutants as molecular baits to capture HIV-1 BaL and IIIB virions. Bars represent the amount of virion-associated p24 captured in replicate experiments.

virion-capture assay than that observed in infectivity assays. All of the other XCL1 mutants showed a slight reduction in virion-capture capacity relative to the unmutated chemokine, suggesting a suboptimal accessibility of the bead-immobilized mutants for interaction with the viral envelope.

Electrostatic interactions play a key role in the anti-HIV activity of XCL1. To further establish the importance of electrostatic interactions in the antiviral activity of XCL1, we produced an additional mutant in which the essential lysine at position 42 was replaced by another positively charged amino acid, arginine (K42R). Strikingly, we observed no loss of function with the K42R mutant in inhibition of either HIV-1 IIIB or BaL infection (Fig. 3A). Furthermore, virion capture of HIV-1 BaL or IIIB was unaffected by the K42R mutation, whereas it was completely lost with the K42A mutation (Fig. 3B). Altogether, these results are compatible with a model whereby the interaction between XCL1 and gp120 is primarily electrostatic in nature.

Structural basis for the selective anti-HIV-1 activity of the alternatively folded, all-β XCL1 conformer. The results obtained by alanine scanning mutagenesis in both infection and virion-capture assays identified five basic residues as key determinants of the antiviral activity of XCL1. When such residues were mapped on the NMR solution structure of the alternative, all-β sheet XCL1 dimer (Ltn40), the conformation associated with antiviral activity (2), we observed that four of them—R18, K42, R43, and K46—are clustered to form a large (~350 Å^2) solvent-exposed, positively charged surface within each subunit of the dimer (Fig. 4A). Thus, the two symmetry-related clusters, which are separated by ~25 Å, likely represent the main interacting domains for HIV-1 gp120. In contrast, the fifth residue, R35, is located on the opposing face of the Ltn40 dimer. Of note, R9 and R23, which have been identified as key residues for the GAG-binding activity of XCL1 (3; Fox et al., unpublished), are located outside the large basic cluster and point

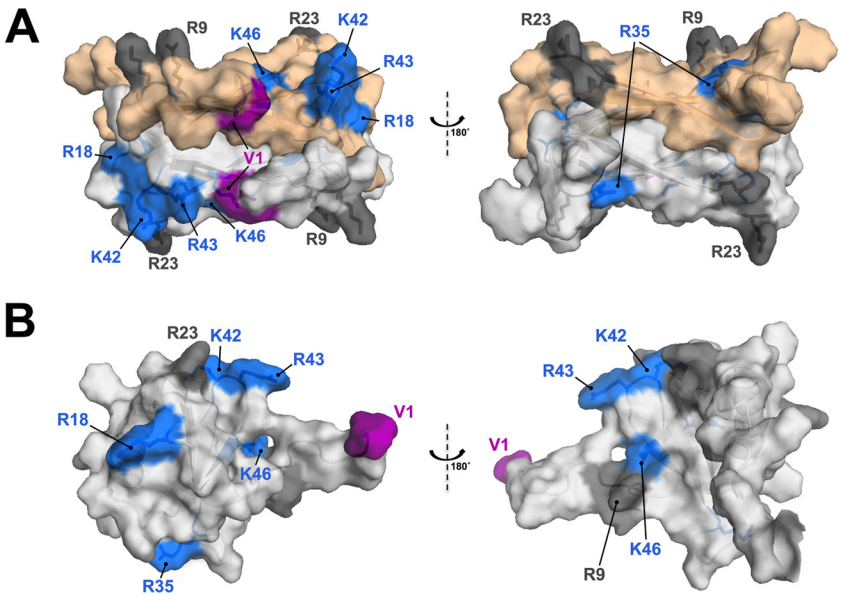

FIG 4 Structural basis for the selective anti-HIV-1 activity of the alternatively folded, all-β XCL1/lymphotactin conformer. (A) Mapping of critical residues for anti-HIV-1 activity on the surface of the alternative, all-β XCL1 structure (Ltn40; dimer), as derived from NMR studies. Basic residues involved in antiviral function are highlighted in blue, while other residues probed by mutagenesis are shown in dark gray, and the N-terminal V1 is shown in purple. The NMR structure was derived from Protein Data Bank code 2JP1. (B) Mapping of critical residues for anti-HIV-1 activity on the surface of the classic chemokine-like folded XCL1 structure (Ltn10; monomer) with the same coloring as in panel A. The NMR structure was derived from Protein Data Bank code 1J9O.

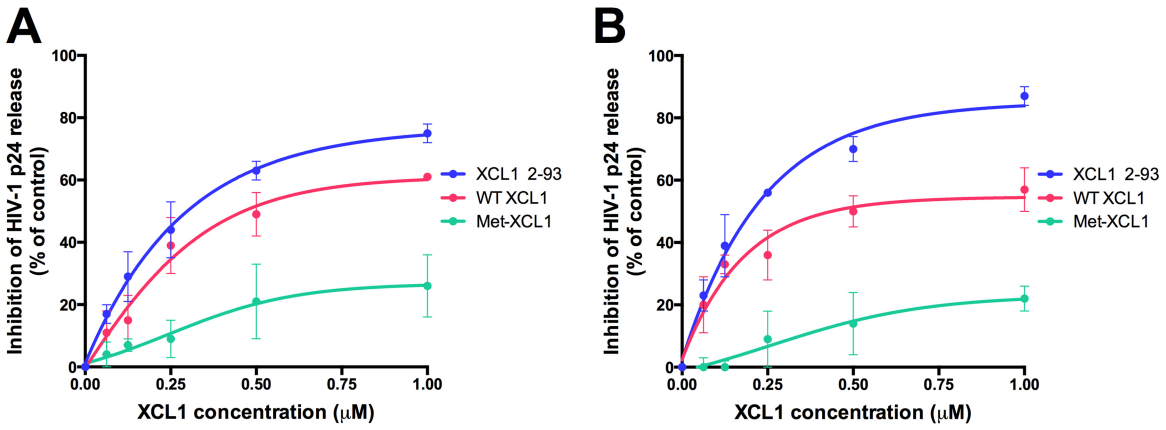

FIG 5 Influence of the N-terminal region on the antiviral activity of XCL1. Inhibitory effect of XCL1 variants with altered N termini against HIV-1 BaL (A) and IIIB (B) as assessed in TZM-bl infection assays. Two commercial variants of XCL1—namely, XCL1 2-93, in which valine 1 was removed (blue), and Met-XCL1 (green)—were compared to wild-type XCL1 (red).

away from the putative HIV-1-interacting surface (Fig. 4A). Strikingly, when the same residues were mapped on the classic chemokine conformation of XCL1 (Ltn10), which is inactive against HIV-1, we found that the 350Å^2 basic surface is not formed, with R18 and K46 located at a distance of 25.7Å and 17.6Å, respectively, from R43 (Fig. 4B). Altogether, these observations identify the large basic cluster formed by R18, K42, R43, and K46 as the primary gp120-interactive site, providing a structural basis for the selective antiviral activity for the alternatively folded, all-β XCL1 conformer.

Influence of the N-terminal region on the antiviral activity of XCL1. In the all-β XCL1 dimer, the N-terminal residue of each subunit (V1, highlighted in purple in Fig. 4A) is positioned in proximity to the basic cluster that we identified as the putative HIV-interactive site, adjacent to K46. Since previous studies have shown the influence of chemokine N termini on anti-HIV activity (14–16), we assayed the antiviral activity of two commercial XCL1 variants which contain altered N-terminal sequences: Met-XCL1 (R&D Systems), which bears an additional methionine before V1, and XCL1 2-93 (Peprotech), in which V1 was removed. As illustrated in Fig. 5, XCL1 2-93 exhibited an increased activity against both R5 (BaL) and X4 (IIIB) HIV-1 strains compared to the wild-type protein, whereas Met-XCL1 showed a decreased anti-HIV function. The data shown represent infection assays in TZM-bl, while similar results were obtained with infection assays performed in primary PBMCs (data not shown). Due to the proximity of the N-terminal valine to the putative HIV-interactive site, it is presumable that the addition of a bulky hydrophobic residue (methionine) might interfere with the interaction of XCL1 with the HIV-1 envelope via steric hindrance or disruption of the local protein conformation.

DISCUSSION

In this study, we sought to investigate the structure-function relationships in XCL1, a metamorphic chemokine that we recently identified as a novel anti-HIV endogenous factor (2). Since we previously demonstrated that the antiviral activity is selectively associated with the alternatively folded, all-β conformation of XCL1, which binds GAGs with high affinity but not the cognate receptor, XCR1, a major aim of our study was to elucidate whether the same structural determinants are involved in the HIV-inhibitory and GAG-binding activities of this chemokine. Through alanine mutagenesis, we identified five solvent-exposed basic residues (R18, R35, K42, R43, and K46) that play a pivotal role in the antiviral activity of XCL1. We observed a good concordance between the results obtained in infection assays and virion-capture assays, further reinforcing our model of HIV-1 inhibition mediated by direct interaction of XCL1 with the external HIV-1 envelope glycoprotein, gp120 (2). The only other chemokine thus far reported to block HIV-1 via direct interaction with the viral envelope is CXCL4/PF4 (11), another protein that displays high-affinity interaction with negatively charged proteoglycans (17, 18). Since the antiviral activity and the structural features of these chemokines are somewhat similar, their interactive surfaces with gp120 may also be overlapping, at least in part, although comprehensive structure-function studies on CXCL4 have yet to be performed.

With regard to the GAG-binding activity of XCL1, previous studies evaluated the effect of alanine substitutions in wild-type XCL1, which is a metamorphic protein. These studies identified two residues, R23 and R43, as critical for heparin-binding affinity (3). However, recent mutagenesis studies were performed with the stabilized, all-β XCL1 variant (W55D), the same used in the present study, leading to the identification of a larger group of residues involved in GAG-binding function (Fox et al., unpublished). Comparison of these data to our scanning analysis indicates that despite a clear overlap between their structural determinants, the antiviral and GAG-binding functions of XCL1 can be uncoupled. Indeed, residues R9 and R23, which are implicated in GAG-binding activity, do not seem to play a role in the anti-HIV activity of this chemokine. Likewise, K46, which is strongly implicated in direct binding of XCL1 to gp120, does not appear to be involved in the GAG-binding activity of XCL1. Nevertheless, our data suggest a model whereby the interaction between XCL1 and gp120 is primarily electrostatic in nature, similar to the interaction with GAGs. This concept was further corroborated by the results obtained with an arginine mutant of the key residue K42, which unlike the alanine mutant, maintained full functional competence in both virus inhibition and virion capture assays.

Analysis of the NMR structure of alternatively folded, all-β

XCL1 conformer revealed that four of the basic amino acid residues involved in the antiviral function are clustered at one end of the β-sheet. Together with the flexible N terminus, these basic side chains and their symmetry-related counterparts from the opposing subunit form an extensive positively charged region that spans the dimer interface and is likely to constitute the primary gp120-interactive site of this chemokine. A specific role for the only residue located outside the major cluster, R35, remains to be established. In striking contrast, this surface is not formed in the classic chemokine-like fold of XCL1, which is inactive against HIV-1, where only K42 and R43 are adjacent to each other. This striking discrepancy in three-dimensional folding of the two conformers provides a structural explanation for the selective antiviral activity of the all-β sheet Ltn40 dimer conformation of XCL1.

The nature of the XCL1-interactive surface in HIV-1 gp120 is still unknown at present. However, in light of the evidence presented here supporting an electrostatic interaction between XCL1 and gp120, we postulate that the interactive surface on gp120 encompasses a region with exposed negative charges. In the case of CXCL4, competition studies using a panel of anti-gp120 monoclonal antibodies of defined specificity have suggested that the CXCL4-binding site partially overlaps with the footprint of VRC03 (11), an antibody directed primarily against the CD4-binding site but with potential contacts also in the V2-V3 variable loop region toward the trimer apex (19). In this respect, we recently reported that the V2 loop is posttranslationally modified by sulfation of tyrosine residues (20), while others have described sulfation of gp120 carbohydrates (21), all of which may contribute negative charges potentially involved in interactions with basic soluble proteins, such as XCL1 and CXCL4. Analysis of surface electrostatic charges and *in silico* docking experiments to identify regions complementary to the putative gp120-binding site of XCL1 are complicated by the remarkable degree of N-glycosylation that decorates gp120, which is only partially and inaccurately represented in recent crystal structures of the HIV-1 envelope trimer (22, 23). Indeed, glycosylation is responsible for 50% of the total molecular mass of gp120 (24), and diversity of the glycan shield exerts a significant influence on gp120 antigenicity (25). Negatively charged surfaces have been characterized in the early crystal structures of HIV-1 gp120 in complex with CD4 (26), and more recently changes in the net negative charge of the fourth variable (V4) domain of gp120 were shown to influence HIV-1 coreceptor usage (7). Furthermore, analysis of gp120 proteins contained in the AIDSVAX B/E vaccine indicated that the producer cell can influence the net surface negative charge of gp120, and consequently the antigenicity of the proteins, which could in turn influence susceptibility to neutralization (27). Preliminary data from our laboratory indeed demonstrated a dramatic reduction of XCL1 activity against HIV-1 pseudoviruses produced in continuous cell lines, compared to virus produced in primary cells which displays a more physiological glycosylation pattern (C. Guzzo et al., unpublished data). Therefore, we postulate that the anti-HIV activity of XCL1 may include interactions with the highly variable glycan shield, in addition to negatively charged amino acid side chains, and a major focus of our future work will be to delineate the precise interaction of XCL1 with a wide range of gp120 glycoform variants.

We also examined the contribution of the N-terminal region of XCL1 because of the proximity of the first residue (V1) to the basic cluster that we identified as the major HIV-interactive surface in the all-β XCL1 conformer. We observed that two N-terminal modifications present in different commercial preparations of recombinant XCL1 influence the antiviral activity. Previous studies have shown that the addition of a methionine at the N terminus of CCL5/RANTES and CXCL12/SDF-1 altered their biological activity and specifically their HIV-1 inhibitory function, leading to reduction of inhibition for Met-RANTES and enhancement of HIV-1 inhibition for Met-SDF-1β (14, 15, 28). In the case of XCL1, we observed a reduction of antiviral activity with Met-XCL1 and a gain of function with the 2-93 variant, indicating that the presence of a bulky structure at the N terminus may sterically hinder the interaction with gp120 or interfere with the correct folding of the HIV-interactive basic cluster. Notably, these observations caution that the source of recombinant XCL1 may influence the interpretation of functional studies.

The identification and characterization of novel endogenous anti-HIV factors, such as XCL1, may open new perspectives for research on pathogenesis, treatment and prevention of HIV-1 infection. Our identification of the structural determinants of antiviral activity in XCL1 provides a template for the rational design of effective HIV-1 entry inhibitors mimicking the active site of this chemokine.

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