Identification of CXCR4 Domains That Support Coreceptor and Chemokine Receptor Functions

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Chemokines are a soluble peptide family that modulate the immune response by virtue of their chemoattractive and signaling properties (see reference 51 for a review). Chemokines are divided into two major classes, CC and CXC, based on the spacing of their two highly conserved Cys residues. Stromal cell-derived factor 1 (SDF-1) is an 8-kDa CXC chemokine originally isolated from a bone marrow stromal cell line (60) that activates a wide variety of primary cells and cell lines (2, 9, 48). The importance of this chemokine in immunomodulation, organogenesis, and hematopoiesis has been highlighted by the characterization of SDF-1 and CXCR4 knockout mice (47, 59, 69). Both exhibit significant developmental abnormalities, indicating that chemokines can play a critical role during development in addition to their well-characterized role in the mature immune response.

The importance of SDF-1 to human disease has also been highlighted by the discovery that a naturally occurring polymorphism in the SDF-1 gene is correlated with slower progression to AIDS in human immunodeficiency virus (HIV)-infected individuals (66). While the mechanism behind this observation has yet to be fully explained, the only known receptor for SDF-1, CXCR4 (8, 48), is the major HIV type 1 (HIV-1) coreceptor used by X4 strains of the virus (also referred to as T-tropic or syncytium-inducing strains) (5, 27). Interaction between the viral envelope (Env) protein and a coreceptor such as CXCR4 triggers conformational changes in Env that lead to membrane fusion and entry of the viral genome into the host cell cytoplasm. SDF-1, like other coreceptor ligands, can block HIV-1 from utilizing CXCR4 and entering a cell (8, 48). Since the emergence of X4 strains of HIV-1 in vivo is correlated with a rapid decline in CD4+ T cells in infected individuals (42), the availability of CXCR4 to X4 strains of HIV-1 in vivo is likely to be a major factor determining the protective effect of the SDF-1 mutation.

Despite its protective effects, the ability of SDF-1 to block HIV-1 coreceptor utilization is variable, often weak, and largely dependent on the Env protein of HIV-1 that mediates the fusion process (62). Previous studies have shown that the extracellular loops (ECLs) of CXCR4, particularly the first and second ECLs (ECL1 and ECL2), are important for coreceptor activity, but the results also suggest that Env-CXCR4 interactions can vary depending on the virus strain studied (10, 40, 50). The identification of small-molecule antagonists of CXCR4 and readily selected strains of HIV-1 that can resist inhibitor challenges highlights the flexibility of Env and the need to understand the interaction of ligands with CXCR4 to design more effective antiretroviral agents (20, 21, 38, 46, 49, 56). Recent advances in detecting direct Env interactions with CCR5 have enhanced our understanding of the role of the chemokine receptors in fusion (37, 41, 52, 61, 67, 68), but direct interactions of X4 EnvS with CXCR4 have been difficult to study (4, 34, 39, 43).

To better understand the basis for SDF-1-mediated disease protection, SDF-1-induced signaling, and CXCR4 coreceptor function, we analyzed the interactions between SDF-1, HIV-1 Env, and CXCR4. We identified a principal SDF-1 binding determinant on the CXCR4 amino terminus and a distinct region...
on ECL-2 of CXCR4 that mediates activation of the receptor by SDF-1. Our data are consistent with models proposed by Crump et al. and Heveker et al. in which the RFFSH motif of SDF-1 (amino acids 12 to 17) mediates binding to the amino terminus of CXCR4, while the first two amino acids of SDF-1 (Lys-Pro) mediate activation of CXCR4 by interacting with ECL2 (16, 36). HIV-1 fusion required regions of CXCR4 that overlapped the binding and activation regions used by SDF-1, but the ability of CXCR4 to signal was clearly distinct from its ability to function as a coreceptor, similar to CCR5. Binding of the gp120 subunit of X4 Env to CXCR4 was dependent on a conformationally complex structure on CXCR4. However, several mutants of CXCR4 that exhibited no detectable binding of X4 gp120 could still function as fusion coreceptors, suggesting that binding of monomeric gp120 to CXCR4 does not necessarily predict coreceptor activity.

Materials and Methods

CXCR4 chimeras and mutants. The CXCR4 chimeras used in this study and the pT4 plasmid encoding human CD4 have been described previously (40). Chimeras were produced by joining CXCR2 and CXCR4 clones in the pcdNA3 vector and are named as described on the parental receptor from which the extracellular domains are derived. For example, 2444 contains the amino terminus of CXCR2 and the first, second, and third ECLs of CXCR4. In brief, chimeras were joined at the following CXCR4 residues: 2444 (Gly-64), 2442 (Ile-243), 2442 (Ile-243), 2442 (Ile-243), 2442 (Ile-243), and 2442 (Ile-243). Constructs of CXCR4-CXCR2 chimeras and mutants (40) for the ability to bind the chemokines IL-8 and SDF-1, signals upon binding the chemokines IL-8 and SDF-1, but did respond appropriately to these chemokines when the cognate receptor was expressed (Fig. 1). The ability to bind signal in response to SDF-1. CXCR2 (30% identical to CXCR4) signals upon binding the chemokines IL-8 and GROα (1) but does not bind or respond to SDF-1 and does not serve as a coreceptor for HIV-1 (19). We used a Ca2+ mobilization assay to determine which chimeras could signal in response to SDF-1, IL-8, or GROα. COS-SH cells were transiently transfected with the designated chimera, iodinated with the Ca2+-sensitive fluorophore Fura-2/AM, and assayed for Ca2+ mobilization following addition of the indicated chemokine. Untransfected cells did not signal in response to SDF-1, IL-8, or GROα but did respond appropriately to these chemokines when the cognate receptor was expressed (Fig. 1). The concentration of SDF-1 used in this assay, 500 ng/ml (62.5 nM), has previously been shown to stimulate CXCR4 to near-maximal levels (8, 48). The effects of chemokine receptor surface expression levels are accounted for below.

Our results indicate that while the distal amino terminus (the first 27 residues up to the conserved Cys) of CXCR4 was neither necessary (chimera 2444) nor sufficient (4222) for activation by SDF-1, the proximal amino terminus (carboxy terminal to the conserved Cys) near the transmembrane region (2448) was required for SDF-1 activation. Chimera 4442 did not respond to SDF-1, suggesting that the third ECL of CXCR4 may also play an important role in CXCR4 activation. However, we cannot rule out the possibility that the failure of 4442 to signal is due to indirect effects of ECL3 (and adjoining transmembrane domains) substitution on the molecule’s overall conformation. Several additional chimeras were constructed in order to identify the contributions of other regions of CXCR4, such as ECL1 and ECL2, but these mutants (4242, 4424, 2224, 2442b, and 4422) were not expressed on the cell surface.

Flow cytometry. In preparation for flow cytometry (fluorescence-activated cell sorting [FACS]), cells were removed from the plate with 5 mM EDTA in PBS, centrifuged, resuspended in staining buffer (2% [vol/vol] bovine serum albumin in PBS) supplemented with 25% normal rat serum and 25% normal rabbit serum, and placed on ice. Cells were stained with primary monoclonal antibodies (Mabs), washed with staining buffer, and then stained with goat anti-mouse antibody conjugated to either fluorescein isothiocyanate or phycoerythrin (Biosource, Camarillo, Calif.). Fluorescence was monitored on a FACScan instrument with a 15-mW 488-nm blue argon laser (Becton Dickinson, San Jose, Calif.), and data from 10,000 cells were analyzed with CellQuest version 3.0.1 software (Becton Dickinson).

Binding assays. For chemokine binding assays, 5 × 10^5 293T cells transiently transfected by CaPO4, with 4 μg of DNA were resuspended in 75 μl of binding buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 5% bovine serum albumin). Subsequently, 0.1 nM [3H]-SDF-1 (specific activity, 2,200 Ci/mmol; NEN-Dupont) was added to cells in 25 μl of binding buffer for a total volume of 100 μl. Cells were incubated at room temperature for 1 h. Unbound radioactivity was removed by filtering cells through Whatman GF/C filters presoaked in 0.3% polyethylenetimine (Sigma) and washing them two times with 4 ml of wash buffer (50 mM HEPES [pH 7.4], 500 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 5% bovine serum albumin). Filters were counted in a Wallac 1470 Wizard gamma counter.

Env binding assays were performed similarly to SDF-1 binding assays except that binding buffer did not include NaCl. The inclusion of NaCl in Env binding assays eliminated detectable Env binding while inclusion of NaCl in SDF-1 binding assays was required for specific binding to CXCR4. BHR and HXB gp120s were produced by using vaccinia virus as previously described (23) and was >90% pure, as demonstrated by Coomasie blue staining. MN gp120, produced via baculovirus by ImmunoDiagnostics, was obtained through the NIH AIDS Reagent Repository. Five to 20 μg of each protein was iodinated by using Iodogen (Pierce) to specific activities of 5.8 μCi/μg (HXB), 1.7 μCi/μg (BH8), and 3.4 μCi/μg (MN).

Infection Studies. Viral stocks were prepared as previously described (11, 15) by transfecting 293T cells by CaPO4, with plasmids encoding the HXB2 or NL4-3 env and the NL4-3 luciferase virus backbone (pNL-Luc-E−R−). The resulting supernatant was stored at −80°C. For infection, U87-MG cells were plated in 24-well plates that were transfected with the desired plasmid (1.5 to 2 μg of each). Medium was changed after 4 h, and cells were allowed to express overnight. Cells were infected the next day with 100 μl of viral supernatant in a total volume of 500 μl in the presence of 8 μg of DEAE-dextran per ml. Cells were lysed at 3 days postinfection by resuspension in 150 μl of 0.5% Triton X-100, 1 mM EDTA, 500 μl of the resulting lysate was assayed for luciferase activity in a Wallac Microbeta scintillation and luminescence counter, using a luciferase assay kit from Progen. All values were within the linear range of luciferase detection.

Results

CXCR4 domains required for SDF-1-induced signaling. To understand how the chemokine SDF-1 and its cognate receptor CXCR4 interact, we tested a panel of previously described CXCR4-CXCR2 chimeras and mutants (40) for the ability to bind and signal in response to SDF-1. CXCR2 (30% identical to CXCR4) signals upon binding the chemokines IL-8 and GROα (1) but does not bind or respond to SDF-1 and does not serve as a coreceptor for HIV-1 (19). We used a Ca2+ mobilization assay to determine which chimeras could signal in response to SDF-1, IL-8, or GROα. COS-SH cells were transiently transfected with the designated chimeras, iodinated with the Ca2+-sensitive fluorophore Fura-2/AM, and assayed for Ca2+ mobilization following addition of the indicated chemokine. Untransfected cells did not signal in response to SDF-1, IL-8, or GROα but did respond appropriately to these chemokines when the cognate receptor was expressed (Fig. 1). The concentration of SDF-1 used in this assay, 500 ng/ml (62.5 nM), has previously been shown to stimulate CXCR4 to near-maximal levels (8, 48). The effects of chemokine receptor surface expression levels are accounted for below.

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SDF-1 requires residues in ECL2 and second intracellular loop of CXCR4 for signaling. To identify specific residues of CXCR4 that contribute to SDF-1-induced signaling, we used site-directed mutants of CXCR4 (65). We focused on ECL2 because the second ECLs of both CXCR4 and CCR5 make major contributions to HIV-1 coreceptor activity (7, 10, 38, 40) and, in the case of CCR5, to chemokine binding specificity (53). Since SDF-1 and the V3 loop of X4 Envs (implicated in coreceptor interaction [13]) are highly basic, our mutants focused on negatively charged residues within this domain. CXCR4-QAAN changes a conspicuous stretch of negatively charged amino acids, Glu-Ala-Asp-Asp (EADD), in ECL2 to the residues Gln-Ala-Ala-Asn (QAAN). When tested in Ca\(^{2+}\) mobilization assays (Fig. 2A), mutant CXCR4-QAAN failed to signal, highlighting the role of ECL2 residues in SDF-1-mediated signal transduction. Another mutation of an acidic residue in ECL2, D193K (Asp 193 changed to Lys), had no effect on CXCR4 signaling.

Important cytoplasmic residues of CXCR4 that contributed to SDF-1-mediated signal transduction were also identified. The Asp-Arg-Tyr motif (DRY box) is highly conserved among G-protein-coupled receptors, and its mutation in well-studied receptors such as rhodopsin, the α- and β-adrenergic receptors (28–30, 64), and CCR5 (3, 7, 22, 26, 32) eliminates signaling. Mutation of this motif in CXCR4 to Asn-Ala-Ala (NAA) largely eliminated the ability of the CXCR4-NAA mutant to signal (Fig. 2A). We note, however, that CXCR4-NAA may retain at least partial G-protein-coupling capability, as an extremely small Ca\(^{2+}\) mobilization signal was consistently noted. Truncation of the Ser- Thr-rich region of the distal C terminus that contains potential sites of receptor phosphorylation had no effect on the ability of CXCR4 to signal (CXCR4at tail).

**Surface expression and detection limitation of Ca\(^{2+}\) mobilization.** Because adequate cell surface expression of chemokine receptors is a prerequisite for detectable receptor activity, Ca\(^{2+}\) mobilization assays were performed in conjunction with flow cytometry (FACS) on parallel sets of cells (Fig. 1 and 2A). For FACS analysis we used MAbs 12G5, which recognizes the first and second ECLs of CXCR4, 10G2 recognizes the distal amino terminus of CXCR2, and 807 is an isotype-matched (IgG2a) control MAb. Chimera 4222 is not capable of being recognized by any of the antibodies used here but has previously been shown to be expressed on the cell surface (40).

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**FIG. 1.** SDF-1 activation requires the proximal amino terminus and the third ECL of CXCR4. Transiently transfected COS-SH cells were stimulated with the indicated chemokine and assayed for mobilization of Ca\(^{2+}\). All cells were subsequently stimulated with TAP to ensure cell integrity (data not shown). Experiments were repeated at least three times. The names and general structures of chimeric constructs are indicated on the left. The percentage of cells scored as receptor positive (% Gated) and the mean fluorescence of staining (MF; indicated in parentheses) as measured by flow cytometry (FACS) of parallel sets of cells are indicated on the right. MAb 12G5 recognizes the first and second ECLs of CXCR4, 10G2 recognizes the distal amino terminus of CXCR2, and 807 is an isotype-matched (IgG2a) control MAb. Chimera 4222 is not capable of being recognized by any of the antibodies used here but has previously been shown to be expressed on the cell surface (40).
chimera has been confirmed previously by using other antibodies (40).

Due to the reduced expression levels of some chimeras, we addressed the sensitivity of our Ca$^{2+}$ mobilization assay by transfecting limiting dilutions of CXCR4 into COS-SH cells followed by both Ca$^{2+}$ mobilization and CXCR4 surface expression measurements in parallel sets of cells (Fig. 2B). Our results indicated that detection of Ca$^{2+}$ mobilization was at least as sensitive as the ability to detect CXCR4 on the surface of these cells by FACS with 12G5. Thus, mutants of CXCR4 that were expressed on the surface of cells at reduced levels, such as 2444b, can be assayed for Ca$^{2+}$ mobilization with confidence. We conclude that the inability of 2444b, 4442, CXCR4-QAAN, and CXCR4-NAA to produce a measurable Ca$^{2+}$ mobilization response was due not to detection limitations but to their inability to transduce a signal in response to SDF-1.

**SDF-1 requires the amino terminus of CXCR4 for binding.**

The failure of a receptor to signal in response to SDF-1 can be attributed either to its inability to bind SDF-1 or to its inability to be activated by a bound SDF-1 molecule. To distinguish between these possibilities, we analyzed the ability of the same panel of chimeras and mutants to bind iodinated SDF-1. To maximize sensitivity, we used transiently transfected 293T cells, which are capable of high levels of transient expression. The low levels of endogenous CXCR4 (estimated to be $\leq$200 copies per cell [63]) on 293T cells did not interfere with our analyses. Similar results were also obtained with transiently transfected QT6 cells, a quail cell line that expresses no known chemokine receptors (data not shown). COS-SH cells exhibited high background binding under the conditions used and thus were unsuitable for this analysis. Using limiting dilutions of transfected CXCR4 DNA, we found that SDF-1 binding could be detected even when CXCR4 expression levels were nearly undetectable as measured by FACS analysis with 12G5 (data not shown).

Binding assays performed with CXCR4 mutants and chimeras (Fig. 3A) demonstrated a dependence on the amino terminus of CXCR4. Chimera 2444 exhibited only minimal binding of SDF-1, while chimera 2444b was unable to bind SDF-1. These results suggest that the amino terminus of CXCR4, par-
particularly the region after Cys-28, is critical for SDF-1 binding. A chimeric receptor identical to 2444 but using the distal amino terminus of CCR5 instead of CXCR2 produced binding and signaling results identical to that of chimera 2444, thus confirming the role of the distal amino terminus in SDF-1 binding (data not shown). Most notably, CXCR4-QAAN was capable of binding SDF-1 despite its failure to signal, suggesting that these residues in ECL2 are critical for signal transduction mediated by SDF-1. Homologous competition assays (Fig. 3B) indicated that our conclusions are not based on widely varying affinity differences. Calculated $K_I$ values, as derived by the method of Swillens (45, 58), for CXCR4, QAAN, 4442, and 2444 were 85, 68, 37, and 38 nM, respectively.

HIV-1 coreceptor utilization of CXCR4 is independent of the ability of CXCR4 to signal or to bind SDF-1. We have previously used a subset of the mutants presented here to map the coreceptor utilization of CXCR4 by HIV-1 in a cell-cell fusion assay (40). Here we extended this analysis by using a virus infection assay and by correlating our results with the regions of the receptor required for SDF-1 binding and signaling and gp120 binding (below). The ability of our chimeras and mutants to support viral entry was assessed in an assay using recombinant virions that express luciferase after integration and that can be pseudotyped with a desired Env (11, 15). For this assay we used transiently transfected human U87-MG cells because of their ability to support viral expression and their high transfection efficiency. Limiting dilutions of transfected CXCR4 DNA demonstrated that coreceptor activity could be detected with this assay even when coreceptor levels were undetectable by FACS (data not shown).

The distal amino terminus was not required for viral entry, since replacement of the distal amino terminus (2444) did not affect the coreceptor activity of CXCR4 (Fig. 4). Further substitution of the amino terminus (2444b) diminished the coreceptor’s ability to support HIV-1 infection, but the reduced surface expression levels of this mutant (<10% of the wild-type level [data not shown]) may account for this result. However, chimera 2244 does support cell-cell fusion with other Envs (40). Residues in ECL2 (CXCR4-QAAN) were extremely important for coreceptor function, as replacement of these few residues diminished the ability of CXCR4 to support HIV-1 entry. Finally, residues in ECL3 also contributed to coreceptor activity, since chimera 4442 supported entry less efficiently than wild-type CXCR4 (Fig. 4). Thus, residues in all four extracellular

FIG. 3. (A) SDF-1 binding requires the proximal amino terminus of CXCR4. 293T cells transiently transfected with the indicated constructs were tested for binding of iodinated SDF-1. Data shown represent the mean and standard error of experiments repeated two to four times. Values for cells transfected with pcDNA3 were considered background and were subtracted from all measurements. Typical values of total bound radioactivity for transfected cells were 20,000 cpm for CXCR4 and 3,000 cpm for pcDNA3. All chimeric constructs were also tested for binding of iodinated GROs, but despite robust binding to CXCR2, iodinated GROs was incapable of binding any of these chimeras above a minimal 10% specific binding (data not shown). (B) Affinity of SDF-1 for CXCR4 variants. A total of $2 \times 10^5$ transiently transfected 293T cells were used for competition binding of iodinated SDF-1 with unlabeled SDF-1. Results are the average of two independent experiments, and values are normalized to binding levels without competition (100%) and with maximum competition (0%). Maximum plateau levels before normalization are represented in panel A. Results were analyzed by nonlinear regression using GraphPad Prism version 2.0 (45).
regions of CXCR4 appear to contribute to coreceptor activity, in agreement with previous analyses of CXCR4 chimeras and mutants by cell-cell fusion (10, 40, 50).

Our infection results also demonstrated that signaling and coreceptor function are independent activities of CXCR4. The CXCR4 mutant CXCR4-NAA, which largely failed to signal, supported HIV-1 entry. Consistent with a previous report (35), treatment of cells with pertussis toxin eliminated detectable signal transduction by CXCR4 (Fig. 2A) but did not eliminate viral entry, integration, or long terminal repeat expression, all of which are required for the final detection of luciferase in this assay. Several CXCR4 mutants that were incapable of binding SDF-1 (2444b) or that did not signal in response to any chemokine ligand (2442, 4442, and CXCR4-QAAAN) still supported HIV-1 virus entry, providing further evidence that SDF-1 binding and CXCR4 activation are independent of CXCR4 coreceptor function.

Direct binding of X4 Envs to CXCR4. Direct binding of HIV-1 Envs to chemokine receptors has been demonstrated for both CXCR4 (4, 34, 39, 43) and CCR5 (37, 41, 52, 61, 67, 68). However, since chemokine receptors do not normally serve as the primary binding receptors for HIV-1, it is not clear what type of contact between Env and the coreceptors is necessary for Env-mediated fusion. Coreceptor mutants that dissociate Env binding from triggering the conformational changes that lead to fusion will be valuable in dissecting the functional domains of CXCR4 and defining their role in virus-membrane fusion.

To address the relationship between the ability of CXCR4 to support Env-mediated fusion and gp120 binding, we adapted the SDF-1 binding assay to detect direct binding of X4 Envs to cells expressing CXCR4 or mutant receptors. We used iodinated gp120s from the X4 HIV-1 strains HXB, BH8, and MN (6, 12). Soluble CD4 (sCD4) was included in all assays except where noted. As shown in Fig. 5, binding of gp120 to cells expressing CXCR4 was observed only in the presence of sCD4, consistent with the conformational changes induced by CD4 that are believed to expose the chemokine receptor binding site on gp120 (52, 54, 55, 61, 67). In addition, binding was observed only when cells expressed CXCR4; we detected no binding to cells expressing CXCR2 or CCR5 (Fig. 6). Binding of the iodinated gp120s to CXCR4-positive cells was inhibited by unlabeled BH8 and MN gp120s but not by the R5 JRFL gp120 (Fig. 5). CXCR4-gp120 binding was also inhibited by SDF-1, ALX40-4C (a CXCR4 antagonist [21]), and a MAb directed against CXCR4 (12G5). Binding was not inhibited by IL-8 or a control mouse MAb (mIgG). In addition, a MAb (D47) specifically directed against the V3 loop of BH8 prevented BH8, but not MN, binding to CXCR4-expressing cells (Fig. 5). Since calcium ions are required for Env-mediated fusion in a post-CD4 binding step (18), we conducted Env binding assays in a modified binding buffer containing no divalent cations and including 10 mM EDTA. These conditions had no effect on gp120 binding, indicating that the requirement of divalent cations for HIV fusion is not at the level of coreceptor binding.

To address the role that gp120 binding plays in coreceptor function of CXCR4, we screened the panel of CXCR4 chimeras and mutants to determine their ability to bind iodinated gp120 (Fig. 6). Our results indicate that detectable binding of X4 Envs to CXCR4 requires nearly all extracellular regions of CXCR4. Even relatively minor changes to CXCR4, such as
D193K, QAAN, and 2444, significantly diminished gp120 binding. This result is consistent with our finding that nearly all regions of CXCR4 contribute to coreceptor function but is surprising since most of these mutants supported HIV-1 infection at some level (Fig. 4). The one mutant that fully supported X4 Env binding, CXCR4Δtail, is expressed at slightly higher levels than wild-type CXCR4 and was capable of binding gp120 accordingly. Thus, detectable binding of monomeric gp120 to CXCR4 does not necessarily correlate with the ability of a coreceptor to support virus infection.

![Graph showing binding of X4 gp120s directly to CXCR4](image1)

**FIG. 5.** Binding of X4 gp120s directly to CXCR4. The X4 gp120s from BH8 and MN were iodinated and used for binding to 4 × 10⁶ transfected 293T cells expressing CXCR4. All conditions contained 100 nM sCD4 except where indicated. Values represent the average and standard error of two to three independent experiments. To best represent the signal-to-noise levels achieved in this assay, only background binding to filters alone was subtracted from values. Raw values of binding and background binding to cells not expressing CXCR4 are presented in Fig. 6. BH8 exhibited high background binding in the presence of cells regardless of blocking or transfection conditions, and thus the minimal binding of BH8 in the presence of cells was 30% of total binding. Blocking agents and concentrations were as follows: JRFL gp120 (R5), MN gp120 (X4), and BH8 gp120 (X4) Env (250 to 500 nM); 12G5 (anti-CXCR4), D47 (BH8-specific anti-V3 loop), and mIgG (pooled mouse IgG) MAbs (10 μg/ml); IL-8 (CXCR2 ligand) and SDF-1α (CXCR4 ligand) chemokines (100 nM); EDTA (10 mM); and ALX40-4C (anti-CXCR4 antagonist) (5 to 10 μM).

![Graph showing multiple regions of CXCR4 are required for detectable binding of X4 HIV-1 gp120s](image2)

**FIG. 6.** Multiple regions of CXCR4 are required for detectable binding of X4 HIV-1 gp120s. Radiolabeled HXB, BH8, and MN gp120 proteins were used for binding to transiently transfected 293T cells as for Fig. 5. Cells were transfected with the constructs indicated, and background values of binding to cells transfected with pcDNA3 vector alone were subtracted from all measurements. Values represent the average and range of two independent experiments. Constructs have been tested two to four times. Representative raw values for binding to cells containing CXCR4, cells transfected with pcDNA3, and binding to the filter alone were 3,300, 1,600, and 800 cpm for HXB (42,000 cpm added), 2,600, 1,500, and 900 cpm for BH8 (100,000 cpm added), and 7,500, 3,200, and 2,700 cpm for MN (80,000 cpm added). HXB and BH8 are nearly identical clones of the X4 HIV-1 strain IIIB that were prepared and tested completely independently but yielded nearly identical results. For measurement of steady-state kinetics, the proportion of radioligand bound (2 to 9%) is within the optimal range for linear detection of radioligand binding (<10%). Values for binding to membrane-bound CD4 were two- to threefold higher than values for binding to CXCR4 in the presence of sCD4 (data not shown). Radiolabeled JRFL gp120 control exhibited no significant binding to CXCR4 despite robust binding to CCR5 (data not shown).
these chimeras to determine if their activation is quantitatively comparable to that of the wild type, we note that similar effects are well documented in the literature and have been observed with other chemokine receptors. For example, multiple CXCR2 (1) and CCR2 (53) chimeras that exhibit only minimal detectable binding nonetheless signal robustly in response to cognate chemokine ligands, suggesting that detection of high-affinity binding is not absolutely required for signal transduction.

Our results are consistent with a previously proposed two-site model of chemokine-chemokine receptor interaction in which the amino terminus of the chemokine receptor plays a major role in the initial binding of the chemokine, while interaction of the chemokine with the loops of the receptor transmits an activation signal (1, 17, 31, 44, 57). The recent determination of the nuclear magnetic resonance structure of SDF-1 and the accompanying analysis of SDF-1 mutants (16) and of SDF-1-derived peptides (36) provides a model for the interaction of SDF-1 and CXCR4 that complements our current work. Crump et al. showed that SDF-1 binds to CXCR4 by using the RFFESH motif at amino acids 12 to 17 of SDF-1 and subsequently mediates activation of CXCR4 with the first two amino acids of SDF-1 (Lys-Pro) (16). Heveker et al. used a peptide-based strategy to reach very similar conclusions about the functional structures of SDF-1 (36). These two complementary studies of SDF-1 suggest that the two amino-terminal residues of SDF-1 are absolutely critical for signaling, that additional residues in the amino terminus distal to the CXC motif (residues 3 to 8) also contribute to signaling, and that residues proximal to the CXC motif that are focused near positions 12 to 14 (RFF) are critical for SDF-1 binding.

By analogy to other chemokine receptors such as CXCR2, both Crump et al. and Heveker et al. speculate that the primary binding event of SDF-1 occurs at the amino terminus of CXCR4 and that the activation of the receptor occurs through a pocket formed by the loops of CXCR4 (16, 36). In conjunction with these SDF-1 mapping data, our data suggest a model in which the binding of SDF-1 to CXCR4 involves SDF-1 residues R12, F13, and F14 binding directly to the CXCR4 amino terminus, with the proximal amino terminus of CXCR4 playing an especially critical role. The cumulative data also suggest that activation of CXCR4 occurs, at least in part, by contact of SDF-1 residues K1 and P2 with ECL2 of CXCR4. Additional biophysical evidence to confirm this model of SDF-1–CXCR4 interaction is clearly required.

Previous studies have demonstrated that signaling by the chemokine receptor CCR5 is not required for coreceptor function (3, 7, 22, 26, 32), but with the exception of a study that included pertussis toxin in an infection (35), we are not aware of similar studies that eliminate the ability of other coreceptors to signal. We eliminated CXCR4 signaling by altering a predicted G-protein-coupling motif (CXCR4-NAA), by chemical uncoupling of G-protein interaction (pertussis toxin), and by creating mutants that are unable to mediate SDF-1-signal transduction (2444b, 2442, 4442, and CXCR4-QAAN). Nevertheless, most of these modifications did not eliminate coreceptor function. Our analysis has thus separated the abilities of CXCR4 to bind SDF-1, to signal in response to SDF-1, and to act as a coreceptor for HIV-1.

Using virus infection assays, we found that HIV-1 Env utilized a conformationally complex structure involving each of the major extracellular regions of CXCR4 for coreceptor function, in agreement with our previous results using a cell-cell fusion assay (40). The contribution of many regions of CXCR4 to coreceptor function implies that a highly conformational structure created by all extracellular regions of CXCR4 inter-
acts with Env. We addressed the possibility that the failure of some coreceptor mutants to support viral fusion is due to their inability to bind Env. The ability to divide coreceptor function into two discrete steps, Env binding and Env triggering, would help identify important chemokine receptor structures that mediate Env conformational changes and would increase our understanding of the fusion mechanism of HIV. By adapting the conditions of chemokine binding, we established a reliable and specific binding assay for detecting X4 Env binding to CXC4R. While this assay is not as robust as similar assays using R5 Env, multiple controls, including an Env-specific MAb, Env proteins of different coreceptor tropisms, a CXC4-specific MAb, and CXC4R antagonists and agonists, demonstrated the specificity of this assay.

We found that monomeric gp120 binding to CXC4R did not correlate with the ability of CXC4R to support Env-mediated fusion. Several CXC4R mutants and chimeras that efficiently supported virus infection were either diminished in the capacity to bind gp120 or completely unable to do so. We have obtained similar results for R5 gp120 binding to CCR5, in which even small perturbations of the CCR5 protein can completely disrupt detectable gp120 binding without strongly affecting co-receptor activity (reference 24 and our unpublished results). Since CD4 serves as the primary receptor for HIV-1 Env, a strong interaction of gp120 with CXC4R may not be required for coreceptor function. Alternatively, oligomeric Env may interact more strongly with CXC4R than the monomeric gp120 molecules used in this study. In addition, the interaction of Env with CXC4R may be followed rapidly by conformational changes in Env that lead to membrane fusion, making even a low-affinity interaction essentially irreversible in the context of virus infection. The dissociation of co-receptor binding of Env and coreceptor fusion activity is a step toward understanding the molecular basis of how the chemokine receptors function as fusion coreceptors.

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