Identification of Residues of CXCR4 Critical for Human Immunodeficiency Virus Coreceptor and Chemokine Receptor Activities

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CXCR4 is a G-coupled receptor for the stromal cell-derived factor (SDF-1) chemokine, and a CD4-associated human immunodeficiency virus type 1 (HIV-1) coreceptor. These functions were studied in a panel of CXCR4 mutants bearing deletions in the NH2-terminal extracellular domain (NT) or substitutions in the NT, the extracellular loops (ECL), or the transmembrane domains (TMs). The coreceptor activity of CXCR4 was markedly impaired by mutations of two Tyr residues in NT (Y7A/Y12A) or at a single Asp residue in ECL2 (D193A), ECL3 (D262A), or TMII (D97N). These acidic residues could engage electrostatic interactions with basic residues of the HIV-1 envelope protein gp120, known to contribute to the selectivity for CXCR4. The ability of CXCR4 mutants to bind SDF-1 and mediate cell signal was consistent with the two-site model of chemokine-receptor interaction. Site I involved in SDF-1 binding but not signaling was located in NT with particular importance of Glu14 and/or Glu15 and Tyr21. Residues required for both SDF-1 binding and signaling, and thus probably part of site II, were identified in ECL2 (Asp187), TMII (Asp237), and TMVII (Glu288). The first residues (2–9) of NT also seem required for SDF-1 binding and signaling. A deletion in the third intracellular loop abolished signaling, probably by disrupting the coupling with G proteins. The identification of CXCR4 residues involved in the interaction with both SDF-1 and HIV-1 may account for the signaling activity of gp120 and has implications for the development of antiviral compounds.

Chemokines are a family of polypeptides promoting the recruitment of various types of leukocytes to sites of inflammation and to secondary lymphoid organs (1, 2). They selectively bind and activate receptors coupled to heterotrimeric G proteins (3, 4). The number and spacing of amino-terminal cysteines allows classification of chemokines into subgroups, C, CC, CXC, and C3XC. Stromal-cell derived factor (SDF-1)† is a CXC chemokine with chemotaxin activity for lymphocytes, monocytes, and their progenitor cells (5, 6), consistent with a role in lymphocyte homing and hematopoiesis (7–9). It has a single known receptor designated CXCR4 (10, 11). Gene inactivation experiments in mice also suggest that CXCR4 and SDF-1 are involved in the embryonic development of several organs, and in particular the brain, heart, and blood vessels (8, 9, 12). In contrast to most other chemokines and their cognate receptors, SDF-1 and CXCR4 are constitutively expressed in many tissues (13–16), which is in agreement with their broad spectrum of biological functions.

Besides its SDF-1 receptor activity, CXCR4 has been identified, along with the chemokine receptor CCR5, as a cell entry portal for the human immunodeficiency virus (HIV-1) (reviewed in Refs. 17–19). Interaction of the HIV-1 surface envelope glycoprotein (gp120) with CXCR4 or CCR5 is necessary to trigger the molecular events that eventually result in virus-cell fusion and infection. In most cases, the contact of gp120 with another membrane component, CD4, is required for a functional interaction with CXCR4 or CCR5, which are therefore viewed to be CD4-associated HIV-1 coreceptors. The cell tropism of HIV-1 strains is in large part governed by the selectivity of gp120 for CXCR4 or CCR5, itself dependent upon the sequence of variable domains, and in particular the V3 loop (20–24). Viral strains using CXCR4 are less frequently isolated than strains using CCR5, until advanced stages of infection (25, 26). Their emergence could have a role in the onset of immune deficiency, possibly by allowing HIV-1 replication in more cell types (16).

Low concentrations of SDF-1 can efficiently block HIV-1 infection mediated by CXCR4 (10, 11). This antiviral effect of SDF-1 is due not only to steric hindrance but also to the down-regulation of CXCR4 at the cell surface by induction of endocytosis (27). Antiviral strategies based on blocking the interaction of HIV-1 with coreceptors are now envisioned (18, 28). To this end, information about the interaction of CXCR4 and CCR5 with their chemokine ligands, and with gp120, is clearly needed.

The structural elements of CXCR4 that mediate the interaction with gp120 or with the chemokine ligands have not been precisely defined. Studies with mutant or chimeric receptors have assigned the HIV-1 coreceptor activity to the extracellular domains, such as the amino-terminal domain (NT) and the second extracellular loop (ECL2) (29–34). Although interaction with gp120 can activate CXCR4 (or CCR5) under certain conditions (35–37), mutations in the intracellular domains that uncouple these receptors with G proteins have no apparent effect on HIV-1 entry (33). It appears therefore that chemokine receptors are used by HIV-1 as docking sites at the cell surface, and not for their ability to transduce a cell signal. Experiments with chimeric receptors also showed that the amino-terminal...
domain of CXCR4 was sufficient for efficient binding of SDF-1, while signaling involved other extracellular domains, and in particular ECL2 (31, 33). These results are compatible with a two-site model for the interaction of SDF-1 and CXCR4 (38), initially developed for the C5a chemoattractant and its receptor (39). According to this model, a discrete region in the amino-terminal domain of the receptor (site I) binds the chemokine with selectivity and high affinity, which then favors its interaction with a pocket formed by the extracellular loops and the membrane-spanning domains (site II). Activation of the receptor requires contact of the chemokine with key residues of site II.

Here we have tested a panel of CXCR4 mutants, comprising deletions in the amino-terminal domain and substitutions of amino acids in the extracellular and transmembrane domains, for their ability to mediate infection of CD4+ cells by two genetically divergent HIV-1 strains, and for their capacity to bind SDF-1 and subsequently mediate a cell signal. On the basis of these experiments, we were able to identify residues of CXCR4 that are involved in these different activities.

EXPERIMENTAL PROCEDURES

Chemokines, Cell Lines, and HIV-1 Strains—Chemically synthesized SDF-1 (275–354) was purchased from Bachem. A gift from F. Barre-Sinoussi (Institut Pasteur, Paris, France) was used. SDF-1a (275–354 µCi/µg) was obtained from NEN Life Science Products. The cell lines COS, HEK293T, and U373MG-CD4 (41) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. U373MG-CD4 are stably transfected with a HIV-inducible β-galactosidase reporter gene (LTR-lacZ). HIV-1 stocks were derived from supernatants of HeLa cells transfected with a cloned LAI provirus (42), or of CEM cells chronically infected with the NDK strain (43). Titers were 105 infectious units (I.U.) per ml for LAI, and ~106 I.U./ml for NDK.

CXCR4 Plasmids and Transfections—The wild-type (WT) and mutant CXCR4 cDNAs were subcloned downstream to the cytomegalovirus promoter gene (LTR-lacZ) or pRc/CMV (Invitrogen). Site-directed mutagenesis was performed on single-stranded templates and checked by sequencing. The position of amino acid substitutions and deletions is depicted in Fig. 1. Mutants Δ2–9, Δ4–36, and N11Q (29), mutants in the ECL2 (30) and in TM, were deleted in mutants Δ2–9, Δ4–36, and N11Q. This deletion has no influence on HIV-1 infection, or on SDF-1 binding and subsequent extracellular events. On the other hand, a 310–354 deletion has no influence on HIV-1 infection, or on SDF-1 binding and subsequent cell signal

Safer inhibition of infection by SDF-1, U373MG-CD4 cells were detached with trypsin 24 h after transfection and seeded in 96-well trays (~104 cells/well). After 24 h, cells in duplicate wells were infected with 10 I.U. of HIV-1 NDK, in the presence of the indicated SDF-1 concentrations. After another 24 h, cells were lysed with 1% Nonidet P-40 and 1% Triton X-100. The cell lysate was centrifuged at 105,000 × g for 20 min, and the supernatant was assayed for luciferase activity. Background was determined by subtracting the luciferase activity in cells infected with SDF-1 from the luciferase activity in cells infected with SDF-1 and HIV-1 (105 I.U./well). Cell counts of 200 were obtained by extrapolation from randomly selected fields. To assess inhibition of infection by SDF-1, U373MG-CD4 cells were detached with trypsin 24 h after transfection and loaded with 500 µCi/ml 3H-thymidine, which was added at a concentration of 0.5 µCi/ml to each well. After 24 h, the supernatant was collected, and 3H-thymidine incorporation was measured in a scintillation counter. The ability of CXCR4 vectors to confer infectivity to CD4− cells was assessed by transfecting CXCR4 vectors in 125I SDF-1-binding cells, and by infection with HIV-1. Fluorescence-activated cell sorting (FACS) analysis was performed to determine the percentage of infected cells. The cell surface expression of wild-type (WT) and mutant CXCR4 vectors and seeded 24 h later in 35-mm glass plates. After another 24 h, cells were loaded with the fluorescent dye fura-2 (Molecular Probes, 3 µM) in saline buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 1.2 unit/ml heparinase III, 1 µM CaCl2, 1 µM MgCl2), then lysed in phosphate-buffered saline with 2% Nonidet P-40, and total γ-radioactivity was counted. In each experiment, counts obtained in cells transfected with pRc/CMV were subtracted as nonspecific SDF-1 binding. Results were then adjusted for the relative cell surface expression of CXCR4.

RESULTS

Surface Expression of CXCR4 Mutants—Our mutagenesis of CXCR4 sought to address the functional role: (i) of the NH2-terminal extracellular domain (NT), by deletions of variable length (Δ2–9, Δ10–20, Δ4–20, and Δ4–36) and by replacing one or several residues, usually with alanine; (ii) of all the charged amino acids in ECL and TM, by individual substitutions with neutral residues; and (iii) of the third intracellular loop (Δ3) by three overlapping deletions. The position of these different mutations and deletions is depicted in Fig. 1. Besides charged residues, mutations in NT addressed the role of Tyr7, Tyr12, and Tyr17, which are potential sites of sulfation (50), and of the potential N-glycosylation site Asn11, mutated alone or with Asn17 in ECL2.

The cell surface expression of wild-type (WT) and mutant CXCR4 was monitored in simian COS cells by flow cytometry. Transfected cells were stained with the 12G5 mAb, which recognizes a conformational epitope in ECL2 (29, 30). Similar results were obtained with the two mAbs, except when mutations disrupted the 12G5 or 6H8 epitope (Fig. 2A and other data not shown). Staining with 12G5 was markedly reduced for cells transfected with the Δ4–36 deletion mutant, while the Δ10–20 and Δ4–20 deletions had a lesser effect (~50% of WT). These deletions probably affect the transport and/or stability of CXCR4 at the cell surface. For all other CXCR4 mutants, the extent of staining was at least 75% of that of WT, suggesting that the overall conformation of the receptor was not significantly altered.

Mutations in CXCR4 Impairing HIV-1 Entry—The ability of CXCR4 mutants to mediate HIV-1 entry in the presence of CD4 (coreceptor activity) was tested in U373MG-CD4 cells. These results showed that the ability of CXCR4 mutants to mediate HIV-1 entry in the presence of CD4 (coreceptor activity) was tested in U373MG-CD4 cells. These results showed that the ability of CXCR4 mutants to mediate HIV-1 entry in the presence of CD4 (coreceptor activity) was tested in U373MG-CD4 cells. These results showed that the ability of CXCR4 mutants to mediate HIV-1 entry in the presence of CD4 (coreceptor activity) was tested in U373MG-CD4 cells.
human astrogliaoma cells must be transfected with a functional coreceptor in order to be permissive to HIV-1 entry (23, 51).

Fig. 2, B and C, show the results of infections performed in parallel with two genetically distant HIV-1 strains, LAI (clade B) and NDK (clade D).

A markedly reduced efficiency of infection by the two HIV-1 strains (<25% of WT CXCR4) was observed upon mutation of two tyrosine residues in the NT domain (Y7A/Y12A). The Y21A mutation also reduced infection by both strains tested (<50% of WT in both cases). All other CXCR4 mutants tested (except Δ4–36) were able to mediate infection by at least one HIV-1 strain with relatively high efficiency (>50% of that of WT), again suggesting that the mutations did not disrupt the overall conformation of the receptor. As we already reported (29), the Δ4–36 mutant cannot mediate NDK infection, despite being a relatively efficient coreceptor for LAI, given its reduced cell surface expression. Mutations D171N in TMIV, D193A in ECL2, and E288A in ECL3 markedly reduced NDK infection, but had lesser effects for LAI. By contrast, the mutations D97N in TMII, D262A in ECL3, and E288Q in TMVII mainly affected infection by NDK. Several other mutations reduced the efficiency of infection by one or the other HIV-1 strain, but to a lesser extent.Mutation of the potential N-glycosylation sites Asn11 and Asn176 had no apparent effect on HIV-1 entry. Overall, our results confirm the importance of residues in NT and ECL2 for the HIV-1 coreceptor activity, but also point to the role of negatively charged residues in the predicted membrane-spanning domains.

**Competition of SDF-1 with Anti-CXCR4 mAbs**—The SDF-1 chemokine can prevent binding of the 12G5 or 6H8 mAbs to CXCR4, and can inhibit HIV-1 infection mediated by the CXCR4 coreceptor (27, 47). These properties were used to address indirectly the ability of SDF-1 to bind to CXCR4 mutants. COS cells transfected with WT or mutant CXCR4 were stained at 4 °C with the 12G5 or 6H8 mAb, in the presence of 200 nM SDF-1. For WT CXCR4, this concentration resulted in 70–80% inhibition of 12G5 or 6H8 binding (data not shown). Table I shows the inhibition of 12G5 or 6H8 binding to cells expressing CXCR4 mutants, relative to cells expressing WT CXCR4. Overall, similar results were obtained in competition assays with the 12G5 and 6H8 mAbs. Almost no inhibition of 12G5 binding (11% of WT), and a limited inhibition of 6H8 binding (35% of WT), was observed for cells expressing the Δ10–20 mutant. SDF-1 also competed rather inefficiently with the 12G5 mAb when cells expressed the Δ4–20 or the Δ4–36 mutant (20 and 36% of WT, respectively), but not the Δ2–9 mutant (>70% of WT). Efficient binding of SDF-1 therefore seems to require residues beyond position 9 in NT. In this domain, mutations at Glu14 and Glu15, or at Tyr21 markedly reduced sensitivity to SDF-1 in assays with the 12G5 mAb (~30% of WT), while their effect was lesser with the 6H8 mAb (50–60% of WT). A reduced effect of SDF-1 (<50% of WT) was also observed for the D97N (TMII) and E288Q (TMVII) mutants. Other mutations in NT (for example, D22A) or in ECL1 (K110A) had a lesser effect. These experiments suggest that residues in NT are critically required for efficient interaction between CXCR4 and SDF-1. This interaction could also be impaired by two out of four mutations in membrane-spanning domains, while few of the mutations in extracellular loops had detectable effect.

**Inhibition of HIV-1 Infection by SDF-1**—Infection of U373MG-CD4 cells expressing WT or mutant CXCR4 with HIV-1 (NDK strain) was performed in the presence or absence of 200 nM SDF-1. This concentration inhibits infection mediated by the WT CXCR4 by 70–80% (Fig. 3). The inhibitory effect of SDF-1 on infection mediated by mutant CXCR4, relative to WT CXCR4 is shown in Table I. All of the NT deletions tested (Δ4–20, Δ10–20, and Δ2–9) reduced sensitivity to the antiviral effect of SDF-1 (23 to 40%). The Δ4–36 mutant is not a functional NDK receptor and could not be tested. In general, HIV-1 infection mediated by CXCR4 mutants with amino acid substitutions in the extracellular domains was efficiently blocked by SDF-1 (>50% of WT), suggesting that ligand binding was relatively conserved. This was in particular the case for
Mutations at residues Glu14-Glu15 and Tyr21, previously found to be important for interaction with SDF-1 in the mAb competition assay. By contrast, markedly lower antiviral effect of SDF-1, suggesting inefficient binding to CXCR4, was observed for the D187A mutant (40% of WT), and for the D97N and E288Q mutants (10% of WT in both cases). These results were confirmed in dose-response experiments (Fig. 3). The SDF-1 concentration yielding 50% inhibition of infection (EC50) was 50 nM for WT CXCR4, and for the E14A/E15A mutant. The EC50 of SDF-1 was higher when cells expressed the Y21A (200 nM), and moreover the D187A, E288Q, D97N, and NT deletion mutants (500 nM). These experiments confirmed the importance of the acidic residues Asp187 in ECL2, Asp97 in TMII, and Glu288 in TMVII for efficient interaction with SDF-1. Mutations at these residues, or the D2–9 deletion, resulted in resistance to the antiviral effect of SDF-1, but they had a lesser effect on the competition of SDF-1 with mAbs. The opposite was observed upon substitutions in NT, such as E14A/E15A and Y21A, while deletions D4–20 or Δ10–20 had an important effect in both assays. Among other experimental differences, competitions with mAbs were performed at 4 °C (1 h), while infections were performed at 37 °C (24 h). Only in the latter case could SDF-1 induce CXCR4 endocytosis. However, this phenomenon did not seem to play a significant role in our experiments, since SDF-1 completely blocked infection mediated by the N11Q mutant, which has a complete deletion of the COOH-terminal domain preventing receptor endocytosis upon ligand binding (27). A more likely explanation for discrepancies between the two types of assays is that competition with mAbs requires a high affinity interaction of SDF-1 with CXCR4, and is therefore highly dependent upon the integrity of site I in NT, while competition with HIV-1 requires interaction of SDF-1 with other residues of CXCR4, probably corresponding to site II.

Radiolabeled SDF-1 Binding—The ability to bind SDF-1 was directly assayed for the deletion mutants (Δ4–36, Δ4–20, Δ10–20, and Δ2–9) and for the E14A/E15A, Y21A, D97N, D187A, and E288Q mutants identified in the previous competition assays. Three CXCR4 mutants apparently able to bind SDF-1 in these assays, E179A, D181A, and D182G, were also included, as the corresponding ECL2 residues are part of a Glu-Ala-Asp motif (EADD) presumed to be required for activation of CXCR4 by SDF-1 (33). Human HEK293T cells transfected with CXCR4 were left in
contact for 90 min with trace amounts of $^{125}$I-labeled SDF-1 (0.2 nM), and cell associated radioactivity was measured. Results were adjusted to account for the relative surface expression of CXCR4 mutants, and for the nonspecific binding. The E179A, D181A, and D182G mutations had no apparent effect on radiolabeled SDF-1 binding (Fig. 4). The other mutant CXCR4 tested bound SDF-1 less efficiently than WT CXCR4, with relative efficiency ranging from 20 to 30% for the NT deletion mutants, or the Y21A, D97N, and E288Q mutants, to 50–60% for the D187A mutant. Thus, mutations that impaired SDF-1 binding in indirect competition assays also reduced SDF-1 binding in a direct assay.

Effects of CXCR4 Mutations on Activation by SDF-1—The activation of WT or mutant CXCR4 expressed in COS cells was detected by a rise in intracellular Ca$^{2+}$ concentration. This parameter was monitored in situ in several cells of a randomly selected field. A marked response to SDF-1 (100 nM) was observed for the WT CXCR4, and for the Δ10–20, E14A/E15A, Y21A, E179A, D181A, and D182G mutants (Fig. 5). There was a low intensity Ca$^{2+}$ response, with very few positive cells for the Δ4–36, Δ4–20, and E288Q mutants, and no detectable signal for the Δ2–9, D97N, and D187A mutants. Residues Glu$^{14}$-Glu$^{15}$ and Tyr$^{21}$ were therefore required for SDF-1 binding but not for signaling, and could be part of the SDF-1 binding site I of CXCR4. Residues Asp$^{97}$, Asp$^{187}$, and Glu$^{288}$ were required both for SDF-1 binding and signaling, and could be part of the site II. The finding that the first residues of NT are required for signaling in response to SDF-1 and for binding was less expected. This point will be further discussed below.

TABLE I

| CXCR4          | Inhibition of anti-CXCR4 mAb binding | Inhibition of NDK infection |
|---------------|-------------------------------------|----------------------------|
|               | 12G5      | 6H8  | %   | %     |
| WT            | 100       | 100  | 100 |
| Δ4–36         | 36.3      | NT$^a$ | NT$^b$ |
| Δ2–9          | 75.4      | 71.3 | 39.8 |
| Δ10–20        | 11        | 35   | 39.2 |
| Δ4–20         | 29.1      | 20.4 | 23.2 |
| E2A           | 101.2     | 103.3 | 113.3 |
| Y7A           | 69.2      | 88.5 | 9.0  |
| Y7A/Y12A      | 61.2      | 82   | 57   |
| D10A          | 82.5      | 86.2 | 105.8 |
| N11Q          | 98.2      | 88.4 | 96.5 |
| EE14–15AA     | 27.6      | 57   | 102.3 |
| D20A          | 65.7      | 76.8 | 107.3 |
| Y21A          | 30.4      | 52.1 | 60   |
| D22A          | 59.4      | NT$^a$ | 75.9 |
| K25A          | 112.5     | NT$^a$ | 130.3 |
| E26A          | 63.5      | 78.5 | 91.1 |
| REE30–32AAA   | 86.6      | 74.8 | 55.7 |
| K38A          | 104       | 98.8 | 113.8 |
| K110A         | 59.8      | 81.3 | 88.2 |
| N11Q/N176D    | NT$^a$    | 76.4 | 102.2 |
| E179A         | NT$^a$    | 95   | 75   |
| D181A         | NT$^a$    | 110.4 | 67.7 |
| D182G         | NT$^a$    | 107.4 | 71.9 |
| R183A         | 117       | 114.6 | 70.8 |
| D187A         | 63.3      | 72.6 | 39.3 |
| R188A         | 140.5     | 64.6 | 69.4 |
| D193A         | 86.8      | NT   | NT$^b$ |
| D262A         | 86.2      | NT   | 65.2 |
| E288A         | 117       | NT   | 122.7 |
| K271A         | 85.6      | NT   | 91.3 |
| E275A         | 113.2     | NT   | 119.7 |
| E277A         | 73.3      | NT   | 64.3 |
| K282A         | 83.6      | NT   | 52.3 |
| D267N         | 46.3      | 47.6 | 9.7  |
| D84N          | 107.7     | NT   | 67.1 |
| D171N         | 77.8      | NT   | 67.5 |
| E288Q         | 40.9      | 64.6 | 7.7  |

$^a$ Not tested, mutation disrupting the 6H8 or the 12G5 epitope.

$^b$ Not tested, not a functional HIV-1 coreceptor for the NDK strain.

FIG. 3. Inhibition of HIV-1 infection by SDF-1. U373MG-CD4 cells transfected with WT or mutant CXCR4 were seeded in 96-well plates and duplicate wells infected with HIV-1 (~10$^5$ I.U./well, NDK strain), in the presence of indicated SDF-1 concentrations. Infection was monitored after 24 h by assaying β-galactosidase activity in cell lysates with the chlorophenol red-β-galactopyranoside substrate. The readout was absorbance at 575 nm ($A_{575}$), corrected for background by subtracting $A_{575}$ measured in CXCR4-transfected uninfected cells processed in parallel. Efficiency of infection (%) was: 100 × $A_{575}$ in the presence of SDF-1/$A_{575}$ in the absence of SDF-1. Data are shown as average ± S.E. from at least two independent transfections.
Functional Studies of Mutant CXCR4

Role of Third Intracellular Loop in Signal Transduction—

The mutation of an Asp-Arg-Tyr (DRY) motif in the second intracellular loop (i2) of CXCR4 abolished signaling in response to SDF-1 (31, 33). This motif is conserved among chemokine receptors and probably required for their coupling to G proteins (52). Here we have addressed the possible role of the third intracellular loop (i3) in signal transduction by testing three CXCR4 mutants with overlapping deletions of 4 residues (Fig. 1). These mutants were apparently expressed normally at the cell surface (Fig. 6A), and mediated HIV-1 infection with an efficiency similar to WT CXCR4 (data not shown). Infection and reactivity with the 12G5 mAb were fully inhibited by 200 nM SDF-1 (Fig. 6B), indicating that the chemokine binds normally to the Δi3 mutants. Upon contact with 100 nM SDF-1, there was no intracellular Ca2+ rise when cells expressed the Δi3-A mutant bearing the most NH2-terminal deletion (Fig. 6C). A Ca2+ signal in response to SDF-1 was detected in cells expressing the Δi3-B and Δi3-C mutants, albeit relatively weak for Δi3-B. One or several residues of the Ser-His-Ser-Lys motif deleted in the Δi3-A mutant seem therefore critical for the coupling of CXCR4 with G proteins. Moreover, our results confirmed that signaling through CXCR4 is dispensable to HIV-1 entry, at least under these experimental conditions (33).

DISCUSSION

The G protein-coupled receptor CXCR4 and its ligand, the SDF-1 chemokine, seem to participate in apparently diverse biological functions, such as hematopoiesis and the embryonic development of brain or blood vessels. In addition, CXCR4 is a CD4-associated HIV-1 coreceptor, allowing cell entry in numerous cell types and probably playing a major role at the advanced stages of infection. HIV-1 entry can be efficiently blocked by SDF-1 and derived peptides (49, 53), or by other CXCR4 ligands such as bicyclams (54). Dissecting the structural domains of CXCR4 supporting SDF-1 receptor and HIV-1 coreceptor activities can therefore contribute to the understanding of developmental processes and virus entry, and also provide information for the design of novel antiviral drugs. In this study, we have tested a series of mutant CXCR4 for their cell surface expression, ability to mediate infection by genetically distant HIV-1 strains (LAI and NDK), and binding to SDF-1.

Determinants of CXCR4 Supporting Its HIV-1 Coreceptor Activity—

Several studies have pointed to the role of two extracellular domains of CXCR4, NT, and ECL2, in its HIV-1 coreceptor activity (29, 30, 32–34). We had previously reported that deletion of most of NT (Δ4–36), or substitutions in ECL2 (e.g. D193A) impaired the coreceptor activity of CXCR4 for HIV-1 strains such as NDK, while having a lesser effect for the LAI strain (29). In the present study, we identified mutations in CXCR4 impairing the coreceptor activity to a similar extent for both strains tested (e.g. E2A, Y7A/Y12A, or Y21A, all in NT), and mutations predominantly affecting infection by NDK (D171N in TMIV), or LAI (D262A in ECL3, D97N in TMII, and E288Q in TMVII). This confirmed that HIV-1 strains can have different requirements for functional interaction with coreceptors. But we also report the importance of residues in the third extracellular loop and in the membrane-spanning domains of CXCR4.

Several types of amino acids were mutated in the NH2-terminal extracellular domain. Tyrosine residues seem to play a role in coreceptor activity, but charged residues (e.g. Glu14 and/or Glu15) could also be involved. Only charged amino acids were mutated in the extracellular loops and membrane-spanning domains. Several negatively-charged residues apparently required for coreceptor activity were identified. The importance of Tyr residues for HIV-1 coreceptor activity was also documented in the case of CCR5 (55–57). These residues have been proposed to interact with hydrophobic residues of the CCR5-binding site of gp120 (58). Since these gp120 residues are relatively conserved among HIV-1 strains, they could also contribute to the interaction with CXCR4. Alternatively, Tyr residues of CXCR4 could contribute to the coreceptor activity through their sulfation, a post-translational modification resulting in addition of a negatively charged group, as proposed for CCR5 (50).

The selectivity of gp120 for CXCR4 seems due in large part to the accumulation of basic residues in variable loops, in particular V3 (20, 22, 24). The V3 loop is also responsible for the inability of the NDK strain to infect cells via the rat CXCR4 (23), or the human CXCR4 with mutations at Asp193 in ECL2 (30). Electrostatic interactions could therefore take place between basic residues of gp120 and the acidic residues we have identified in extracellular and membrane-spanning domains (Asp97) of CXCR4. Recently, Chabot et al. (34) also reported that mutation of Asp97 markedly impaired the HIV-1 coreceptor activity of CXCR4. This residue, or Glu288 in TMVII are probably relatively close from the cell surface. They may engage electrostatic interactions with a protruding region of gp120, such as the V3 loop, and contribute to its anchoring in a pocket formed by the extracellular domains of CXCR4.

Determinants of the SDF-1 Receptor Activity—

The interaction of SDF-1 with CXCR4 was first addressed in competition assays involving binding of anti-CXCR4 mAbs or HIV-1 infection. A relatively high concentration of the chemokine (200 nM) was used in these assays. CXCR4 mutants whose interaction with SDF-1 was apparently impaired were tested for their ability to bind trace amounts (0.2 nM) of radiolabeled SDF-1 and to mediate a rise in intracellular Ca2+ in response to SDF-1, which indicates receptor activation.

The Glu14 and Glu15 (mutated together) and Tyr21 residues in NT were apparently required for SDF-1 binding but not for cell signaling, consistent with their belonging to the ligand-binding site I of CXCR4. Deletion of residues 10–20 (Δ10–20) also disrupted the putative site I. Aromatic (Tyr) and acidic

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**Fig. 4.** Binding of radiolabeled SDF-1 to cells expressing WT or mutant CXCR4. HEK293T cells in 6-well trays were transfected with CXCR4, incubated for 90 min with 125I-SDF-1 (0.2 nM), and cell associated radioactivity counted in each well. Results were corrected for background, by subtracting radioactivity counts obtained in a parallel transfection with an empty vector, and for cell surface expression of CXCR4 tested in parallel by staining with the 12G5 mAb. Bars represent amount of bound radioactivity for cells transfected with mutant CXCR4, relative to cells transfected with WT CXCR4. Data are shown as average ± S.E. from at least three independent transfections, except for the E179A, D181A, and D182A mutants, only tested once.
residues required for chemokine binding were also identified in the NT of CCR5 (56, 57).

Substitutions of acidic residues in ECL2 (D187A) and in two membrane-spanning domains (D97N and E288Q) affected SDF-1 binding and impaired or abolished receptor activation, suggesting that the corresponding residues contribute to the ligand-binding site II. The first residue of SDF-1 (Lys1) seems crucial for activity (38, 49), and can be envisioned to interact

**FIG. 5.** Intracellular Ca$^{2+}$ fluxes induced by SDF-1 in COS cells expressing WT or mutant CXCR4. Effect of SDF-1 (100 nM) on intracellular Ca$^{2+}$ was monitored in situ in transfected COS cells loaded with the fluorescent dye fura-2. Representative data among several independent experiments are shown.
with the acidic residues of site II, in particular those located in TM domains of CXCR4, or at the interface with ECL1 in the case of Asp$^{97}$. The role of the ECL2, TMII, and TMVII domains of CXCR4 in SDF-1 signaling is consistent with results obtained by Doranz et al. (33) using chimeric receptors. These authors also reported that substitution of three residues at positions 179, 181, and 182 in ECL2 (QAAN mutant) abolished SDF-1 signaling. Here we observed an apparently efficient interaction with SDF-1 and signaling for the E179A, D181A, and D182G mutants. It seems unlikely that mutation of Glu$^{179}$ into Gln, or Asp$^{182}$ into Asn could have different effects, suggesting that the phenotype of the QAAN mutant results from cumulative effects of the mutations.

Deletions encompassing the first residues of NT ($D_2–9$, $D_4–20$, and $D_4–36$) impaired SDF-1 binding and abolished signaling. This suggests that the first residues of NT can have a role in the activation of CXCR4. The assumption that the amino terminus of CXCR4 comprises different functional regions is in line with findings concerning other chemoattractant receptors. For the C5a receptor, although the ligand-binding site was assigned to residues 21–30 in NT, a deletion of residues 1–22 abolished signaling (59), like the $D_2–9$ deletion in our study. The first residues of NT of CXCR4 could be actually part of site II, possibly interacting with the extracellular loops, as has been proposed for the C5a receptor (60). Alternatively, residues 2–9, although not essential for access of the chemokine to site I, may be required for its proper orientation toward site II, and thus for signaling. Notably, a recently described CXCR4 splice variant, CXCR4-L0, only differs in the first nine residues and yields less potent biological effects in response to SDF-1 (61).

In conclusion, the SDF-1 chemokine and the HIV-1 envelope protein gp120 seem to share certain requirements for their functional interaction with CXCR4, including residues not only in different extracellular domains, like NT and ECL2, but also in membrane-spanning domains. For example, a mutation of Asp$^{97}$ in TMII had an effect both on SDF-1 receptor and HIV-1 coreceptor activities. The role of TM residues in the interaction between CXCR4 and gp120 was unexpected and could explain the ability of gp120 to transmit a cell signal via its corresponding coreceptor, CXCR4 or CCR5 (35–37). Efficient inhibition of HIV-1 infection via CXCR4 seems to require interaction of SDF-1 with residues of CXCR4 involved in signaling (site II). Also, the bicyclam AMD3100 efficiently blocks HIV-1 entry via CXCR4 but behaves as an antagonist for this receptor (54). Antiviral strategies aimed at blocking the coreceptor activity of CXCR4 will certainly require drugs that do not interfere with the chemokine receptor activity. Preliminary studies with SDF-1-derived peptides suggest that this goal can be achieved (49).

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![Fig. 6. Effects of deletions in the third intracellular loop of CXCR4.](image-url)
