A Point Mutation That Confers Constitutive Activity to CXCR4 Reveals That T140 Is an Inverse Agonist and That AMD3100 and ALX40-4C Are Weak Partial Agonists*

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CXCR4 is a G protein-coupled receptor for stromal-derived factor 1 (SDF-1) that plays a critical role in leukocyte trafficking, metastasis of mammary carcinoma, and human immunodeficiency virus type-1 infection. To elucidate the mechanism for CXCR4 activation, a constitutively active mutant (CAM) was derived by coupling the receptor to the pheromone response pathway in yeast. Conversion of Asn-119 to Ser or Ala, but not Asp or Lys, conferred autonomous CXCR4 signaling in yeast and mammalian cells. SDF-1 induced signaling in variants with substitution of Asn-119 to Ser, Ala, or Asp, but not Lys. These variants had similar cell surface expression and binding affinity for SDF-1. CXCR4-CAMs were constitutively phosphorylated and present in cytosolic inclusions. Analysis of antagonists revealed that exposure to AMD3100 or ALX40-4C induced G protein activation by CXCR4 wild type, which was greater in the CAM, whereas T140 decreased autonomous signaling. The affinity of AMD3100 and ALX40-4C binding to CAMs was less than to wild type, providing evidence of a conformational shift. These results illustrate the importance of transmembrane helix 3 in CXCR4 signaling. Insight into the mechanism for CXCR4 antagonists will allow for the development of a new generation of agents that lack partial agonist activity that may induce toxicities, as observed for AMD3100.

CXCR4 is the exclusive receptor for stromal-derived factor 1 (SDF-1), a CXC chemokine that has been shown to play a critical role in directed cell migration (1, 2), embryologic development (3–5), and the metastatic spread of mammary carcinoma cells (6). In addition, T-tropic (X4) strains of HIV-1 utilize CXCR4 for target cell entry (7), and this function is blocked by SDF-1 (8, 9) and receptor antagonists (10–13). The clinical significance of CXCR4 has led to intensive characterization of the structural basis for its function and interaction with its physiologic and pathologic ligands, SDF-1 and the gp120 subunit of X4 Env glycoproteins, respectively. These studies have broadly defined the domains of CXCR4 that play a role in HIV-1 infection, ligand binding, and signaling. However, the molecular mechanisms of ligand-induced conformational changes and subsequent signaling remain unknown.

Three CXCR4 antagonists have been described that block infection by X4 strains of HIV-1 and SDF-1 binding, AMD3100 (10), ALX40-4C (12), and T22/T140 (11, 13). Binding of AMD3100 to CXCR4 has been reported to involve residues in transmembrane helix (TM) 4 and extracellular loop 3 (14) and possibly extracellular loop 2 (15). Administration of AMD3100 results in mobilization of hematopoietic stem cells (16), and clinical trials in AIDS patients were discontinued because of cardiac arrhythmias. The importance of CXCR4 as a potential molecular target in HIV-1 infection and breast cancer provides a strong rationale for characterizing the mechanism of the action of CXCR4 antagonists and development of second generation antagonists with more favorable pharmacological properties.

Here we have adopted a Saccharomyces cerevisiae expression system to couple CXCR4 signaling to growth in the absence of histidine (17) and derived a CXCR4-CAM by random mutagenesis. The amino acid substitution that conferred this phenotype involved Asn-119, which is located in TM3, and further mutagenesis revealed substitutions that stabilized inactive or active receptor conformations in yeast and mammalian cells. Characterization of CXCR4 antagonists revealed that T140 is an inverse agonist and that AMD3100 and ALX40-4C are weak partial agonists. Whereas binding studies failed to reveal evidence of changes in extracellular domains in active or inactive variants, the decreased affinity of CAMs for AMD3100 and ALX40-4C signifies a conformational shift, most likely in the hydrophobic core of CXCR4. Variants with autonomous signaling were chronically phosphorylated and internalized. These findings illustrate the importance of TM3 conformation in the rearrangement in the hydrophobic core of CXCR4 that leads to Gα subunit activation and provide insight into the clinical effects of AMD3100. Utilization of GPCR CAMs in pharmaceu-
tactical screening provides an efficient and powerful approach for identification of novel antagonists.

**EXPERIMENTAL PROCEDURES**

**Yeasts Strains and Plasmids**—CXCR4 was functionally coupled to the pheromone response pathway in the S. cerevisiae strain CY12946 that has been described previously (17). Yeast cells were transformed with CXCR4 constructs using the Frozen-EZ Yeast Transformation-II kit (Zymo Research, Orange, CA). Site-directed mutagenesis was performed using a QuickChange kit (Invitrogen).

**Random Mutagenesis**—The open reading frame encoding CXCR4 was amplified in the presence of manganese and dITP by PCR to achieve a final random mutation rate of 0.1–0.3%. This pool was cloned into an engineered plasmid pool, transformed into CY12946 yeast cells, and grown, and plasmid DNA was extracted from these cultures. The plasmid pool was transformed into CY12946 yeast cells, and colonies were selected for growth in medium lacking histidine. Plasmid DNA from these colonies was sequenced.

Analysis of FUS1-HIS3 and FUS1-lacZ Reporter Genes—Yeast strain transformants expressing different CXCR4 constructs were tested for expression of the pheromone-responsive FUS1-HIS3 reporter gene in medium lacking histidine. Recombinant SDF-1 was obtained from Leiner Technologies, Inc. (St. Louis, MO). Cell density was determined from the absorbance at 600 nm. Expression of the FUS1-lacZ reporter gene was determined using a fluorescent β-galactosidase substrate (Molecular Probes, Eugene, OR). Enzymatic activity was determined using standard approaches. The experimental data were normalized using basal β-galactosidase activity of CXCR4-WT.

**Derivation of Stable CHO Transfectants**—Stable CHO cell transfectants expressing CXCR4 variants were prepared for each construct as described previously (18). Transfectants expressing high levels of each mutant were enriched from pools by magnetic sorting with the anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA), and single cell clones with matched levels of cell surface expression were isolated by immunofluorescent sorting with 12G5. 

**Signal Transduction**—For calcium flux experiments, cells were labeled with Fura-2 acetoxymethyl ester (2 μg/ml) (Molecular Probes) as described previously (18). The response to 10−8 M SDF-1, T140, AMD100, ALX40-4C, or 5-hydroxytryptamine (Sigma) was recorded using a spectrofluorometer (F2500, Hitachi, San Jose, CA) as described previously (18). The endogenous serotonin receptor expressed by CHO cells was stimulated with 10 μM 5-hydroxytryptamine as a control for Gα activation in these cells. [35S]GTPγS binding was performed using membrane pellets (7 μg) and 0.5 nM [35S]GTPγS (>1000 Ci/mmol; American Biosciences) for 1 h at 37 °C. Bound isotope was separated by filtration with GF/C membranes (Whatman) and measured by scintillation counting of the washed, dried filters. Basal binding was determined in the absence of agonists, and nonspecific binding was obtained in the presence of 10 μM GTPγS (Sigma). The percentage of stimulated [35S]GTPγS binding was calculated as 100 × (cpm/sample − nonspecific cpm)/(basal cpm − nonspecific cpm). Coupling to Gα subunits was determined by metabolically labeling transfectants in the presence of pertussis toxin (Calbiochem, La Jolla, CA) overnight.

**CXCR4 Phosphorylation**—Phosphorylation of CXCR4 was performed as described previously (19). CHO transfectants were metabolically labeled with [32P]orthophosphate and then stimulated with SDF-1 or phorbol ester. CXCR4 was immunoprecipitated from detergent lysates via its N-terminal Myc tag with 9E10, and labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.

**CXCR4 Internalization**—Stable CHO transfectants were exposed to buffer or 100 nM SDF-1, permeabilized with 20% methanol, and stained with 9E10 by indirect immunofluorescence.

**RESULTS**

**Asn-119 Substitutions Regulate CXCR4 Activation of G Proteins in Yeast and Mammalian Cells**—S. cerevisiae strains in which activation of CXCR4 results in expression of pheromone-responsive HIS3 or lacZ reporter genes were genetically engineered. CXCR4-CAMs were selected from pools of random mutants of the cDNA in this system. Screening of over 105 recombinant events with a mutational rate of 0.1–0.3% yielded clones with different genotypes, all of which contained an A → G substitution resulting in the N119S mutation. Among 26 clones sequenced, no other mutation was found consistently. Expression of CXCR4(N119S) in CY12946 yeast cells complemented the histidine auxotrophy of this strain, indicating that this mutation is sufficient to activate autonomous CXCR4 signaling. Substitution of Asn-119 with neutral, acidic, or basic residues revealed that Ser and Ala, but not Asp or Lys, supported constitutive activation of the pheromone-responsive expression of the FUS1-lacZ reporter gene (Fig. 1A). Exposure of yeast cells expressing CXCR4(N119S) or CXCR4(N119A) to SDF-1 resulted in a small increase over respective unstimulated β-galactosidase levels (Fig. 1A). CXCR4(N119D) demonstrated a minimal increase in β-galactosidase activity to 1 μM SDF-1, and CXCR4(N119K) was refractory to ligand stimulation.

The authenticity of the signaling phenotype of these CXCR4 mutants in yeast was confirmed in stable CHO transfectants selected for matched cell surface expression of the respective variants. Membrane fractions from CXCR4(N119S) or CXCR4(N119A) transfectants exhibited basal [35S]GTPγS binding that was >5-fold that of CXCR4-WT transfectants (Fig. 1B). Exposure of each CAM to SDF-1 augmented Ga subunit activation. CXCR4(N119D) lacked autonomous signaling but demonstrated SDF-1-induced [35S]GTPγS binding similar to that of CXCR4-WT. CXCR4(N119K) lacked signaling activity. Exposure of the transfectants to pertussis toxin blocked the response of CXCR4-WT to SDF-1 and extinguished the elevated basal [35S]GTPγS binding observed in CXCR4(N119S) transfectants (Fig. 1C).

The ability of CXCR4 variants with substitutions of Asn-119 to transduce SDF-1-mediated signaling in calcium mobilization experiments is shown in Fig. 1D. CXCR4-WT transfectants demonstrated a response to 0.03 nM SDF-1 that was of maximum intensity at 10 nM. In transfectants expressing CXCR4(N119S) or CXCR4(N119A), a minimal response was detected following stimulation with 0.3 nM SDF-1, and maximal calcium mobilization was induced at 10 or 100 nM SDF-1, respectively. The magnitude of peak calcium mobilization in CXCR4(N119S) or CXCR4(N119A) transfectants was less than transduced by CXCR4-WT. The calcium response to SDF-1 in CXCR4(N119D) transfectants was similar to that of the WT receptor. In contrast, CXCR4(N119K) transduced a minimal response following exposure to higher SDF-1 concentrations.

**CXCR4 Variants with Asn-119 Substitutions Retain SDF-1 Binding Properties of CXCR4-WT**—Binding experiments were performed to determine whether the active conformation of CXCR4-CAMs affected affinity for SDF-1. Parallel analysis of [32P]SDF-1 binding to stable CHO transfectants with matched levels of CXCR4-WT, CXCR4(N119S), CXCR4(N119A), CXCR4(N119D), or CXCR4(N119K) expression revealed that they bound ligand with similar IC₅₀ values (Table 1). These CXCR4 variants had Myc and His₃ epitope tags at the N and C termini, respectively, and this was associated with a slight decrease in binding affinity for SDF-1 (CXCR4 2 μM; Myc-CXCR4-His₃ 0.3%). All CXCR4 variants with Asn-119 substitutions had similar levels of 12G5 staining (data not shown).

**CXCR4-CAM Is Constitutively Phosphorylated and Down-modulated**—Metabolic labeling experiments were performed to determine whether the CXCR4-CAM is phosphorylated. Whereas CXCR4-WT was not phosphorylated in resting cells, exposure to SDF-1 induced receptor phosphorylation (Fig. 2A). Incubation of CXCR4-WT transfectants with phorbol ester also induced receptor phosphorylation. In contrast, CXCR4(N119S)
FIG. 1. Substitution of Asn-119 in TM3 governs CXCR4 coupling to Go subunits in yeast and mammalian cells. CXCR4 Asn-119 mutants were tested for autonomous and SDF-1-induced activation of the pheromone-responsive FUS1-lacZ reporter gene in S. cerevisiae (A). β-Galactosidase activity from standardized cultures of yeast strains expressing the CXCR4 variants was determined as described under “Experimental Procedures.” CXCR4 Asn-119 mutants were tested for autonomous and SDF-1-induced coupling to Go subunits in stable CHO transfectants (B). [35S]GTPγS binding was performed as described under “Experimental Procedures.” Utilization of Go subunits for SDF-1-induced and CAM signaling was determined by preincubation of CHO transfectants expressing CXCR4-WT or CXCR4(N119S) with pertussis toxin (C). [35S]GTPγS binding was performed as described under “Experimental Procedures.” The values are the means ± S.E. of triplicate samples, and the results are representative of three independent experiments (A–C). The capacity of CXCR4 Asn-119 mutants to transduce SDF-1 signaling was determined by calcium flux assay (D). Stable CHO transfectants were loaded with Fura-2 and exposed to incremental doses of SDF-1 as described under “Experimental Procedures.” The results are representative of two independent experiments. PTX, pertussis toxin.
Constitutive Activity of CXCR4

TABLE I

<table>
<thead>
<tr>
<th>CXCR4 (Myc/His)</th>
<th>Signaling</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; of SDF-1</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
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<tr>
<td>Wild type</td>
<td>SDF-1</td>
<td>19.8 ± 5.8</td>
<td>16.0</td>
</tr>
<tr>
<td>N119S</td>
<td>CAM</td>
<td>19.2 ± 4.5</td>
<td>27.6</td>
</tr>
<tr>
<td>N119A</td>
<td>CAM</td>
<td>10.9 ± 5.1</td>
<td>13.4</td>
</tr>
<tr>
<td>N119D</td>
<td>SDF-1</td>
<td>32.6 ± 0.4</td>
<td>16.6</td>
</tr>
<tr>
<td>N119K</td>
<td>No</td>
<td>7.2 ± 1.7</td>
<td>8.4</td>
</tr>
</tbody>
</table>

* ND, not determined.

**Fig. 2.** CXCR4-CAMs are chronically desensitized. Phosphorylation of CXCR4-WT and CXCR4 Asn-119 substitutions that confer autonomous signaling (Ser) or inactivity (Lys) was determined by metabolic labeling with [32P]orthophosphate and immunoprecipitation (A). CHO transfectants with stable cell surface expression of CXCR4-WT (lanes 1–3), CXCR4(N119K) (lanes 4–6), or CXCR4(N119S) (lanes 7–9) at matched levels were exposed to control buffer (lanes 1, 4, and 7), SDF-1 (lanes 2, 5, and 8), or phorbol ester (lanes 3, 6, and 9), and detergent lysates were immunoprecipitated with 9E10, as described under “Experimental Procedures.” The results are representative of two independent experiments. Down-modulation of CXCR4-WT (panels A and B), CXCR4(N119S) (panels C and D), and CXCR4(N119K) (panels E and F) in the absence (panels A, C, and E) or presence (panels B, D, and F) of SDF-1 was determined by immunofluorescence as described under “Experimental Procedures” (B). The results are representative of two independent experiments.

was phosphorylated in resting cells, and the levels were not altered by exposure to SDF-1 or phorbol ester. The CXCR4(N119K) mutant showed minimal phosphorylation induced by SDF-1 and had low level phosphorylation following exposure to phorbol ester.

Immunofluorescence localization experiments were performed to determine whether CXCR4-CAMs were internalized. CXCR4-WT was localized at the surface of transfectants but was redistributed to cytoplasmic inclusions following stimulation with SDF-1. The CXCR4-WT was present in the cell surface in the absence and presence of SDF-1. CXCR4(N119K) was present on the cell surface in the absence and presence of SDF-1. The CXCR4-WT was localized at the surface of transfectants but was redistributed to cytoplasmic inclusions following stimulation with SDF-1. The CXCR4(N119S) mutant showed minimal phosphorylation induced by SDF-1 or phorbol ester. The CXCR4(N119K) was present on the cell surface in the absence and presence of SDF-1. CXCR4-N119S was present on the cell surface in the absence and presence of SDF-1.

Two Classes of CXCR4 Antagonists: T140 Is an Inverse Agonist, and AMD3100 and ALX40-4C Are Weak Partial Agonists—The effect of CXCR4 antagonists on CXCR4-CAM signaling was determined in yeast and mammalian cells. Exposure of yeast cells expressing CXCR4(N119S) to incremental concentrations of ALX40-4C resulted in increased β-galactosidase levels at 1 μM (Fig. 3A). Incubation with AMD3100 resulted in significant increases in β-galactosidase levels at 100 nM. In contrast, exposure to T140 decreased autonomous signaling in a dose-dependent manner.

The ability of these antagonists to influence CXCR4-CAM signaling was tested in CHO cells stably expressing CXCR4(N119S). Incubation of membrane fractions from CXCR4-CAM transfectants with 1 μM ALX40-4C or AMD3100 increased [35S]GTPγS binding (Fig. 3B). In contrast, exposure to T140 resulted in a dramatic, dose-dependent decrease in [35S]GTPγS binding.

The impact of CXCR4 antagonists on signal transduction by the CAM was further characterized in calcium mobilization experiments (Fig. 3C). AMD3100 or ALX40-4C stimulated a low amplitude of calcium mobilization in CXCR4-WT transfectants. In contrast, exposure of the same cells to T140 resulted in a brief decrease in free cytosolic calcium. All three antagonists inhibited subsequent responses to SDF-1.

Parallel experiments were performed using CXCR4(N119S) transfectants to characterize the effect of these antagonists (Fig. 3C). Exposure to AMD3100 resulted in increased cytosolic calcium levels similar to those elicited by 1 nM SDF-1. The transfectants were refractory to subsequent SDF-1 stimulation. Exposure of the transfectants to ALX40-4C also mobilized cytosolic calcium ions. Further exposure to SDF-1 induced a dampened response that was less than that observed in naive cells. Incubation of CXCR4(N119S) transfectants with T140 induced a transient decrease in free cytosolic calcium and significantly reduced SDF-1-induced signaling. Identical experiments with CXCR4(N119A) transfectants revealed similar results (data not shown). The ability of SDF-1 or AMD3100 to induce signaling in a nontransfectant system was established in THP-1 cells, a primitive monocytic cell line with endogenous CXCR4 expression. As shown in Fig. 3D, exposure to SDF-1 or AMD3100 resulted in calcium mobilization responses similar to those observed in CHO cell transfectants. Exposure to ALX40-4C did not induce a significant calcium flux (data not shown).

A dose response curve for the effect of AMD3100 on transfectants expressing CXCR4-WT or CXCR4(N119S) is shown in Fig. 3E and F. A direct calcium mobilization response was first evident in CXCR4-WT transfectants at 100 nM AMD3100 (Fig. 3E). The response to stimulation with 1 nM SDF-1 was decreased by 100 nM AMD3100 and dramatically inhibited at 1 μM.

Parallel analysis of CXCR4(N119S) CHO transfectants revealed that a calcium flux response was detected following exposure to 1 nM AMD3100 (Fig. 3F). The magnitude of the response was augmented by incremental doses of bicyclam to peak levels at 1.0 μM. Inhibition of SDF-1-induced signaling mediated by the CAM was evident at AMD3100 concentrations of 10 nM and complete at 1 μM.

Conformational Shifts in CXCR4-CAMs Preferentially Affect AMD3100 and ALX40-4C Binding—The ability of T140,
FIG. 3. Classification of CXCR4 antagonists as weak partial agonists (AMD3100 and ALX40-4C) or inverse agonists. The effect of the known CXCR4 antagonists on autonomous signaling by CXCR4(N119S) was determined from basal expression of the pheromone-responsive FUS1-luc2 reporter gene (A). Exposure of standardized yeast cultures to CXCR4 antagonists and analysis of β-galactosidase levels were determined as described under “Experimental Procedures.” The values are the means ± S.E. of triplicate samples. The results are representative of three independent experiments. The effect of the known CXCR4 antagonists on autonomous coupling of CXCR4(N119S) to Gα subunits in mammalian cells was determined by [35S]GTPγS binding as described under “Experimental Procedures” (B). The values are the means ± S.E. of triplicate samples. The results are representative of three independent experiments. The ability of CXCR4 antagonists to induce signaling and inhibit SDF-1-induced activation was determined in calcium mobilization experiments (C). Transfectants expressing matched levels of CXCR4-WT or CXCR4(N119S) were loaded with Fura-2; exposed to control buffer, AMD3100, ALX40-4C, or T140; and then exposed to SDF-1 (1 nM) as described under “Experimental Procedures.” The results are representative of two independent experiments. The ability of SDF-1 or AMD3100 to induce signaling in an hematopoietic cell line that expresses CXCR4 was determined in THP-1 cells (D). THP-1 cells were loaded with Fura-2 and exposed to SDF-1 or AMD3100 as described under “Experimental Procedures.” The results are representative of three independent experiments. The dose-response effect of AMD3100 on SDF-1 (1 nM) stimulation of CXCR4-WT (E) and CXCR4(N119S) (F) was determined as described under “Experimental Procedures.” The results are representative of two independent experiments. 5-HT, 5-hydroxytryptamine; AMD, AMD3100.
AMD3100, and ALX40-4C to displace \(^{125}\text{I}\)SDF-1 binding was determined for CXCR4-WT and the variants with Asn-119 substitutions. \(^{125}\text{I}\)SDF-1 displacement from stable transfectants failed to reveal a difference in the IC\(_{50}\) for T140 binding to WT, CAM, and inactive variants (Table I). Displacement of \(^{125}\text{I}\)SDF-1 binding by AMD3100 was similar in CXCR4-WT, CXCR4(N119D), and CXCR4(N119K) (Fig. 4). In contrast, AMD3100 displacement of \(^{125}\text{I}\)SDF-1 binding to the CXCR4-CAMs was significantly decreased (Fig. 4 and Table I). Parallel studies showed a similar effect with ALX40-4C (Table I).

**DISCUSSION**

Here we demonstrate that Asn-119 plays a critical role in the mechanism of CXCR4 signaling through its regulation of TM3 conformation. Conversion of Asn-119 to Ser or Ala was found to drive the conformational equilibrium of CXCR4 to the active state, manifested by autonomous signaling. In contrast, substitution of Lys for Asn-119 induced a conformation of TM3 that favors the inactive state, rendering CXCR4 unresponsive to SDF-1 binding, rendering CXCR4 unresponsive to SDF-1 without affecting the binding. The autonomous coupling of the CXCR4-CAMs to G\(_{\text{q,16}}\) subunits was augmented by SDF binding, consistent with stabilization of an optimal active conformation. AMD3100 and ALX40-4C were also found to increase the signaling of CXCR4-CAMs and, to a lesser extent, that of CXCR4-WT, indicating that they are weak partial agonists. In contrast, T140 was found to be an inverse agonist that reversed the conformational equilibrium induced by activating mutations to stabilize the inactive form. Although there was no evidence for altered architecture of CXCR4 extracellular domains, significant decreases in the affinity of AMD3100 and ALX40-4C binding provided objective evidence for a conformational shift in the hydrophobic core of the receptor.

The availability of CXCR4-CAMs should provide a powerful tool for high throughput screening for antagonists. Agents that inhibit the WT receptor may increase or reduce the signaling of CAMs, depending on whether the mechanism of action is that of a weak partial agonist, such as AMD3100 and ALX40-4C, or an inverse agonist, such as T140. The effects of AMD3100 and ALX40-4C on CXCR4 signaling were amplified in the CAMs despite a significant decrease in the affinity, presumably because the threshold for receptor activation was diminished in the variants.

Engagement of GPCR by ligand results in a conformational shift of the hydrophobic core and cytoplasmic domains to a state permissive for the formation of a high affinity ternary complex with Go subunits (20). It is predicted that Asn-119 is located midway between the extracellular and cytoplasmic interfaces of the TM3 helix of CXCR4 and is oriented toward the center of the hydrophobic core based on comparison with the rhodopsin crystal structure (21). The N(L/F)YSS motif is highly conserved in TM3 of receptors for CXC chemokines and may play a critical role as a switch that maintains the dynamic conformational equilibrium that regulates coupling to Go subunits. AMD3100 binding has been reported to involve Asp-171, which is predicted to reside in TM4 (14). This supports the interpretation that the binding pocket for this partial agonist involves regions in the hydrophobic core. Thus the decrease in binding affinity of CAMs reflects a conformational shift in this region of CXCR4. The absence of alterations in SDF-1, T140, and 12G5 binding by the CAMs provides evidence that TM3 has sufficient intrinsic flexibility to permit significant internal rearrangement of the hydrophobic core without altering the architecture of extracellular domains.

Constitutively active variants of several GPCR have been described, either as a natural occurrence or as the product of genetic manipulations (22–29). This is the first report of a constitutively active variant of a chemokine receptor. Human herpesviruses encode chemokine receptor orthologs that exhibit autonomous signaling, the Kaposi’s sarcoma herpesvirus (KSHV) (30, 31) and human cytomegalovirus (32). The GPCR encoded by the KSHV (KSHV-GPCR) is genetically related to the chemokine receptor family (30, 31). This receptor displays ligand-independent, constitutive activity that is mediated by the phosphoinositide-specific phospholipase C signaling pathway (33). The KSHV-GPCR binds multiple chemokines that may serve as agonists (34) or inverse agonists (35). The precise mechanism for the autonomous activity of this receptor is unclear, because it differs significantly from even the most closely related genomically encoded receptor at the level of primary structure. It has been shown that charged residues at the interface of TM3 (Arg-143) and TM2 (Asp-83) with the adjacent cytoplasmic interhelical loops influence the constitutive activity of this receptor (36). Whereas CXCR4-CAM signaling in CHO cells was sensitive to pertussis toxin, KSHV-GPCR signaling in endothelial cells involves multiple pathways, including pertussis toxin-sensitive and -insensitive mechanisms (37).

The CXCR4-CAMs demonstrate evidence of chronic desensitization, as has been observed in autonomously active variants of multiple GPCR (23, 25), that involves receptor phosphorylation and internalization. CXCR4 mutants with truncation of phosphorylation sites in the C-terminal tail have been shown to exhibit increased signaling responses, indicative of loss of negative regulatory control mechanisms (19). The finding that CXCR4-CAMs were constitutively phosphorylated provides additional evidence that the cytoplasmic aspect of these receptor variants mimics the active conformation induced by ligand binding, which is recognized by GPCR kinases. Whereas CXCR4 has been shown to be a substrate for protein kinase C (19), the unresponsive CXCR4(N119K) variant appeared to have decreased phosphorylation following ligand stimulation or exposure to phorbol ester. These findings support the interpretation that the architecture of the cytoplasmic aspect of the nonactivated receptor may be poorly recognized by GPCR kinases. The localization of CXCR4-CAMs to perinuclear structures suggests that these receptors are actively internalized in the absence of SDF-1 engagement, although the formal possibility that this distribution is due to disruption of intracellular trafficking cannot be fully excluded. However, CXCR4-CAMs were targeted to the cell surface at levels similar to that of the WT receptor, although a portion may be incompletely trafficked. A transient decrease in cytosolic free calcium levels occurred following exposure of CXCR4-CAM transfectants to
T140, indicating a switch of the receptor to the inactive conformation. AMD3100, ALX40-4C, and T22/T140 have been reported to antagonize the activation of CXCR4 by SDF-1 and to block infection by X4 strains of HIV-1 in vitro (10–13). Whereas AMD3100 and ALX40-4C stimulated the signaling of the CXCR4-CAM in yeast and mammalian systems, T140 inhibited the autonomous coupling to Go subunits in both. The maximum response of CXCR4-WT induced by high concentrations of AMD3100 or ALX40-4C was less than observed with SDF-1; thus these agents are weak partial agonists. Because T140 functions as an inverse agonist, it is likely that it interacts with CXCR4 through a mechanism distinct from that employed by the partial agonists. The evidence that coreceptor signaling by gp120 plays a critical role in viral replication (38) raises the possibility that partial agonists. The evidence that coreceptor signaling by CXCR4 through a mechanism distinct from that employed by the partial agonists. The evidence that coreceptor signaling by CXCR4 through a mechanism distinct from that employed by the partial agonists.

Constitutive Activity of CXCR4