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ACCELERATED COMMUNICATION

Recombinant Human CXC-Chemokine Receptor-4 in Melanophores Are Linked to G_i Protein: Seven Transmembrane Coreceptors for Human Immunodeficiency Virus Entry into Cells

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ABSTRACT

This article describes the transient expression of the CXC chemokine receptor-4 in *Xenopus laevis* melanophores and the resulting functional assay for the endogenous ligand for this receptor stromal cell-derived factor (SDF)-1α. Specifically, it will be shown that SDF-1α produces increased light transmittance in transfected cells that is consistent with the activation of G_i protein. This stimulus pathway is further implicated by the abolition of this response after pretreatment of the cells with pertussis toxin, a known method for the inactivation of G_i protein. The fact that SDF-1α does not produce responses in nontransfected cells and that treatment of the cells with 12G5, an antibody specific for the CXC chemokine receptor-4, eliminates this response indicates that this ligand produces responses by activation of this receptor in these cells. The possible relevance to human immunodeficiency virus (HIV) entry into cells was explored by observing the effects of SDF-1α on HIV-mediated cell fusion. It was found that SDF-1α blocked cell-to-cell fusion (as has been previously reported) at concentrations 1200-fold greater than those required to produce G_i protein mediated responses. The implications of the functional assay to screening for new drugs to block HIV-mediated fusion is discussed.

The discovery that HIV-1 utilizes seven transmembrane receptors as coreceptors for viral fusion has introduced a new target into the therapeutic field of view. Studies on HIV-1 envelope-mediated cell fusion have identified a 46-kDa, integral membrane protein named "fusin" that serves as a cofactor for HIV fusion and entry (Feng *et al.*, 1996). Fusin is a 352-amino-acid, seven-transmembrane receptor, the sequence of which is identical to a previously cloned orphan receptor denoted LESTR (leukocyte-derived seven-transmembrane domain receptor). Sequence comparison has shown that fusin (or LESTR) is a member of the CXC chemokine receptor family with a sequence homology to the CXCR2 receptor (Loetscher *et al.*, 1994; Raport *et al.*, 1996; Wells *et al.*, 1996). The identification of SDF-1α (a CXC chemokine) as a ligand for fusin has led to the suggested classification of this receptor as CXCR4 in the chemokine receptor nomenclature system (Bleul *et al.*, 1996; Oberlin *et al.*, 1996).

This article describes the construction of a receptor assay in which the human CXCR4 couples to G_i-protein in recombinant *Xenopus laevis* melanophores. In these cells, melanosome dispersion can be affected via activation of adenylyl cyclase (Potenza *et al.*, 1992; McClintock *et al.*, 1993) or phospholipase C (Graminski *et al.*, 1993), whereas melanosome aggregation results from the inhibition of adenylyl cyclase (Potenza *et al.*, 1992; McClintock *et al.*, 1993). Melanophore cells contain a wide range of G_α-proteins (Jayawickreme *et al.*, 1994); therefore, the expression of numerous foreign G protein-coupled receptors can be facilitated (Potenza *et al.*, 1992, 1994; Karne *et al.*, 1993; McClintock *et al.*, 1993; Graminski *et al.*, 1993 and 1994; Jayawickreme *et al.*, 1994a, 1994b; Lerner, 1994). Because both states of in-

ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; CXCR*x*, CXC chemokine receptor, where *x* is the receptor number; SDF, stromal cell-derived factor; BSA, bovine serum albumin; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; ED_{50}, dose effective on 50% of the population.

tracellular melanosome distribution (dispersion or aggregation) are easily detectable, various G protein-coupled receptors have been studied by monitoring ligand-mediated melanosome translocation, either by measuring the change in light transmittance through the cells or by imaging the cell response (Potenza *et al.*, 1992, 1994; Karne *et al.*, 1993; McClintock *et al.*, 1993; Graminski *et al.*, 1993 and 1994; Jayawickreme *et al.*, 1994a, 1994b; Lerner, 1994).

We will show that SDF-1α mediates responses through CXCR4 and G_i protein; thus, the assay can be used to study ligand/receptor interaction. Although the implications of this functional linkage to viral fusion are presently unclear, the assay has utility as an indicator of receptor conformational states, and therefore as a CXCR4 screen.

Experimental Procedures

Materials. Leibovitz (L-15) medium was purchased from Sigma Chemical (St. Louis, MO), BSA was obtained from Boehringer Mannheim (Indianapolis, IN) and pertussis toxin was obtained from Calbiochem (La Jolla, CA). 12G5 antibody was generously provided by Jim Hoxie (University of Pennsylvania, Philadelphia, PA). The full-length cDNAs for CXCR4 and SDF-1β were kindly provided by Dr. Christine Power (Glaxo Wellcome, Geneva, Switzerland).

Construction of expression vectors. The full-length cDNA for CXCR4 was cloned by a reverse transcriptase-PCR strategy. The sets of primers used for PCR were designed according to the GeneBank accession number M99293. The full-length cDNA of human SDF-1β was cloned by screening a human spleen cDNA library using the mouse SDF cDNA as a probe (R. B. Furness and C. Power, personal communication, 1997).

CXCR4 was subcloned as a *Hin*dIII/*Xba*I fragment into the melanophore expression vector, pJG3.6 (Graminski *et al.*, 1993). CXCR4 plasmid DNA used for melanophore transfections was prepared by a modification of the triton-lysozyme method and double-banded in CsCl/ethidium bromide equilibrium gradients as described (Davis *et al.*, 1994).

Oligonucleotide primers were designed for PCR amplification of the SDF-1α cDNA corresponding to the mature form of the protein (no signal sequence). One primer included the 5′-untranslated region of the bacteriophage T7 gene10A before the region coding for the amino terminus of SDF-1α. The sequences of primers are listed below: 5′-α: TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAGCCCGTCAGCCTGAGCTACAG; 3′-α: ACGCGTCACTTGTTTAAAGCTTTCTCCAGGTACTC. The SDF-1α PCR fragment was inserted into the plasmid pCRII (InVitrogen, San Diego, CA) forming pCRII/SDF1α. An *Xba*I-*Sac*I fragment was excised and inserted between the *Xba*I–*Sac*I sites in pTX007, forming pTXSDFA9 (Dallas *et al.*, 1992).

Expression and purification of SDF-1α. Strain BL21[DE3] expressing SDF-1α was grown until absorbance was 0.6 at 37°, and induced by 0.25 mM isopropyl β-D-thiogalactoside for 3 hr. SDF-1α was purified to homogeneity using a three-column step procedure. The cell pellets were resuspended in a lysis buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 10 μM 4-amidinophenylmethanesulfonyl fluoride) and disrupted by a French pressure cell (SLM Instruments, Rochester, NY). After a low-speed centrifugation, the pellet was washed with lysis buffer and solubilized in a 8 M urea buffer (8 M urea, 20 mM dithiothreitol, and 1 mM EDTA) overnight. The solubilized material was loaded onto SP Sepharose columns and eluted with the same buffer containing 1 M NaCl. The SDF-containing fractions were identified by SDS-polyacrylamide gel electrophoresis and pooled. After renaturation in a buffer containing 0.1 M Tris·HCl, pH 8.0 and 0.1 mM glutathione, the proteins were loaded onto two tandem HiTrap SP Sepharose columns (Pharmacia Biotech, Piscataway, NJ) and eluted by a NaCl gradient in 25 mM Tris·HCl, pH 8.0. The fractions containing SDF-1α were loaded onto a reversed-phase poros column and eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid. The purified SDF-1α was speed-vacuum-dried to complete dryness, and then resuspended in phosphate-buffered saline (0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, 2.16 g/liter $Na_2HPO_4{\cdot}7H_2O$) and stored at −80° until use.

Functional bioassay. Melanophores were maintained in cell cultures as described previously (Jayawickreme *et al.*, 1994a, 1994b). Transient expression of CXCR4 plasmid DNA in melanophores was achieved after electroporation (Graminski *et al.*, 1993; Jayawickreme *et al.*, 1994). After electroporation, cells were seeded into flat-bottomed, 96-well, tissue culture plates (Falcon Plastics, Oxnard, CA) to a density of 40,000 cells/well in conditioned fibroblast medium and incubated at 27° for 24–48 hr.

The ligand mediated responses in recombinant melanophores were monitored by measuring the change in transmittance using an SLT Spectra plate reader (Austria). For each experiment, media was removed from the plates and replaced with 0.7× L-15/0.1% BSA containing test drugs. Soon after the addition of reagents, the zero time reading (T_i) was obtained. The plates were then placed in the dark and read at appropriate time intervals (T_f). The extent of the response was quantified as $(1 - T_f/T_i)$ (Jayawickreme *et al.*, 1994a; Potenza *et al.*, 1994).

In the studies employing pertussis toxin, the transfected cells were incubated overnight (16–18 hr) in conditioned fibroblast medium with 1 μg/ml of pertussis toxin. Just before the assay, the media was removed and replaced with 0.7× L-15/0.1% BSA containing various reagents and/or drugs. In studies employing 12G5 antibody, the cells were incubated with antibody at a concentration of 16.9 μg/ml in 0.7× L-15/0.1% BSA media for 30 min before assaying.

Viral fusion assay. An HIV-mediated fusion assay was developed based on the coculture of human embryonic kidney 293 cells stably transfected with pHIVΔRT*Bst*EII (Kellam and Larder, 1994), designated 293ΔRT, and 1G5, a Jurkat T cell line containing a long terminal repeat-luciferase gene (Aguilar-Cordova *et al.*, 1994). The 293ΔRT cells express HIV-1 gene products (except reverse transcriptase) and readily induce syncytia formation when co-cultured with $CD4^+/CXCR4^+$ cells (R. Ferris, and L. Boone, unpublished observations, 1997). The 1G5 cell line is susceptible to T cell line tropic HIV and, after infection, expresses luciferase from the transfected HIV long terminal repeat-luciferase reporter gene. Coculture of 293ΔRT cells with 1G5 cells results in fusion and tat-mediated activation of luciferase.

The ability of SDF-1α to block HIV-mediated cell-to-cell fusion was determined by measuring its effect on the activation of luciferase. 293ΔRT cells were plated at 5×10^4 cells/well (96-well cell culture plate) in RPMI 1640, 10% fetal bovine serum and allowed to grow overnight. 1G5 cells were added to a final concentration of 1×10^5cells/well in the same medium containing indicated concentrations of SDF-1α and coculture was continued for 5 hours. Media was removed and cells were lysed with 200 μl of lysis buffer (Luciferase Assay System; Promega, Madison, Wisconsin) for 20 min at room temperature followed by one freeze/thaw cycle. Luciferase activity was measured by incubating 20 μl of lysate with 50 μl of luciferase reagent (Promega) and reading them immediately in a luminometer (ML1000; Dynatech Laboratories, Chantilly, VA). Duplicate samples were averaged.

Results and Discussion

SDF-1α is a CXC chemokine produced by bone marrow stromal cells characterized by a typical four-cysteine motif (Baggiolini *et al.*, 1994). There are two spliced forms of SDF-1, SDF-1α and SDF-1β. The amino acid sequences of SDF-1α and -β are identical for the first 89 amino acids, but SDF-1β has four additional amino acids at the carboxyl ter-

minus. This chemokine is known to have multiple biological activities [e.g., proliferation of B cell progenitors (Tashiro *et al.*, 1993; Nagasawa *et al.*, 1994) and production of lymphocyte migration (Bleul *et al.*, 1996)]. To test the functional activities of SDF-1α, the cDNA for this protein was first cloned and expressed.

A T7 polymerase-based *Escherichia coli* expression system was used to express recombinant SDF-1α. pCRII is a plasmid designed for cloning PCR fragments with an unpaired A residue at each 3′-end. Because this plasmid also has a T7 promoter near the site of fragment insertion, we anticipated that by adding a ribosome binding region to the SDF-1α cDNA before cloning, the T7 promoter could be used to enhance expression. Results of the induction experiments with pCRII/SDF-1α in BL21[DE3] showed that initially (1 hr after induction), SDF-1α was one of the major proteins, but after 3 hr, it was almost totally degraded. We transferred the SDF-1α fragment from pCRII/SDF1α to pTX007, a plasmid we had used successfully for high level expression of a variety of proteins. When induced, BL21[DE3](pTXSDFA9) made SDF-1α to the extent that it was the major protein 1 hr after induction. The protein continued to accumulate and, over time, formed inclusion bodies. A three-step purification procedure was developed including the separation of renatured SDF-1α from denatured proteins. The purified proteins were analyzed on an SDS gel as shown in Fig. 1A. Amino-terminal sequence determination showed that the initiator methionine residue was not removed; therefore, recombinant SDF-1α differs from the native form. The deduced amino acid sequences of SDF-1α are shown in Fig. 1B.

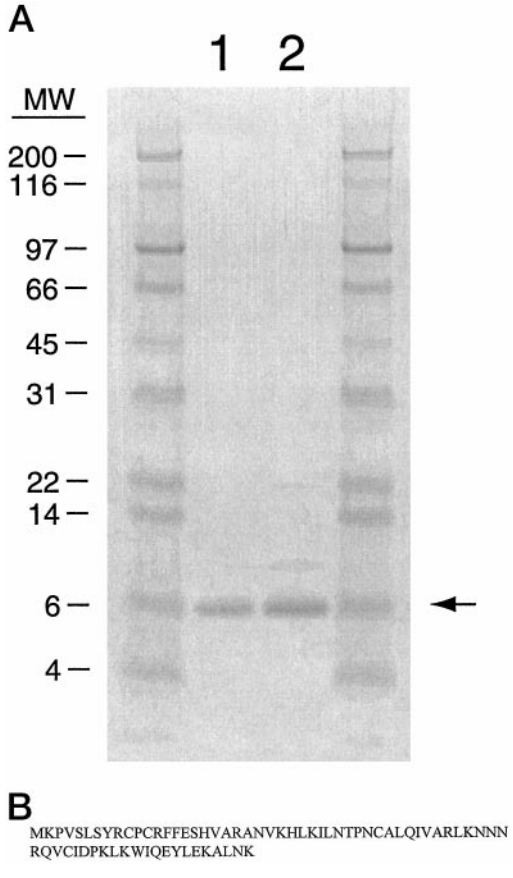

Fig. 1. A, SDS-polyacrylamide gel electrophoresis analysis of purified SDF-1α. SDF-1α was purified as described in Experimental Procedures. One microgram (*lane 1*) or 2 μg (*lane 2*) of purified protein was loaded onto a 4–20% SDS gel and stained with Coomassie Brilliant Blue. The protein molecular mass standards used were myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97 K; serum albumin, 66 K; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 22 kDa; lysozyme, 14 kDa; aprotinin, 6 kDa; and insulin β chain, 4 kDa. B, Deduced amino acids of purified recombinant protein.

SDF-1α has recently been shown to be a ligand for CXCR4 and to produce calcium responses in Chinese hamster ovary cells stably transfected with CXCR4 (Bleul *et al.*, 1996; Oberlin *et al.*, 1996). Accordingly, a functional assay for CXCR4 was established. SDF-1α produced a concentration-dependent aggregation response in melanophores transfected with CXCR4 cDNA. Fig. 2 shows the response to SDF-1α measured at timepoints 30 to 210 min. It can be seen that a steady state with SDF-1α was obtained after 90 min; the concentration producing ED_{50} was found to be 40 pM in the experiment shown. The mean ED_{50} from nine experiments is 53 ± 11 pM. One feature of G_i protein-coupled receptor effects in melanophores is the time-dependence of the response. Specifically, once G_i protein has been activated, the cell must reach a new steady state with respect to the altered cytosolic level of cyclic AMP; the time for this effect can vary. This property of melanophores makes temporal study of G_i-mediated responses important for the determination of the potency of agonists. The changing responses with time probably do not reflect the temporal interaction of SDF-1α and CXCR4, because the ED_{50} does not change after 30 min. Rather, the increased asymptotic response reflects the clearance of endogenous cyclic AMP in the presence of a new setpoint of G_i activation (Potenza *et al.*, 1994). Although CXCR4 is similar to IL-8 receptors (CXCR1, CXCR2), IL-8 did not produce activation of CXCR-4 in melanophores (Fig. 2). No response to SDF-1α was observed in melanophores not transfected with CXCR4 or CXCR1 (data not shown). The involvement of G_i-protein in the response to SDF-1α was suggested by the fact that the ligand-mediated response is

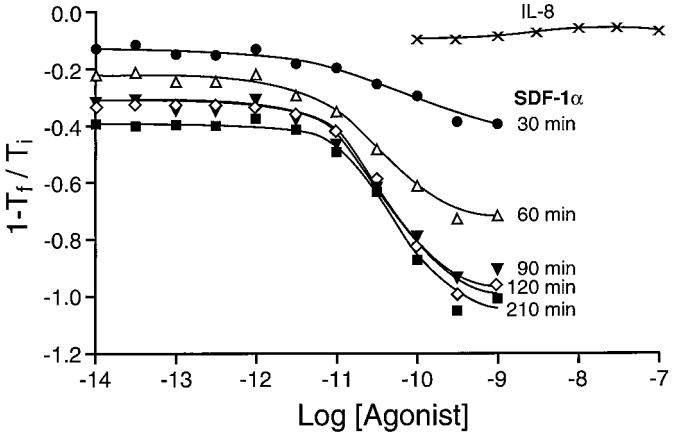

Fig. 2. SDF-1α mediated receptor activity of CXCR-4 transiently transfected into melanophores. Effect of increasing concentrations of SDF-1α (abscissae as log scale) on melanophore responses (ordinates as $1 - (T_f/T_i)$ where T_i and T_f refer to transmittance through the well before and after addition of SDF-1α, respectively). A, Dose-response curves to SDF-1α obtained at various times of incubation: ○, 30 min; △, 60 min; ▼, 90 min; ◇, 120 min; and ■, 210 min. The lack of response to IL-8 in transfected cells also is shown (×).

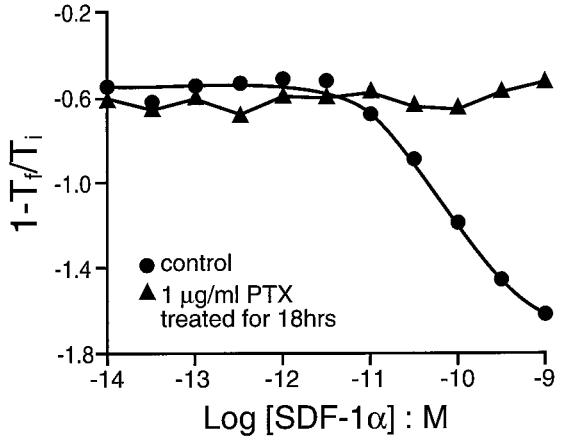

Fig. 3. Effect of pretreatment of melanophores with pertussis toxin on SDF-1α mediated CXCR-4 receptor activity. Ordinates and abscissae as for Fig. 2. Responses shown in the cells pretreated (▲) and not pretreated (●) with pertussis toxin.

abolished in transfected cells treated with pertussis toxin (Fig. 3), a treatment that inactivates G_i protein.

To further examine the interaction of SDF-1α with the CXCR4, experiments were done on melanophores pretreated with 12G5, a monoclonal antibody specific for CXCR4 (Endres *et al.*, 1996). As shown in Fig. 4, pretreatment of transfected melanophores with 12G5 produced an inhibition of the SDF-1α response. The dose-response curve to SDF-1α was shifted to the right by a factor of 100 after treatment with 12G5 antibody.

SDF-1α has been shown to block T cell trophic HIV-1 cell-to-cell fusion and infection (Oberlin *et al.*, 1996). We wanted to determine the relative potency of SDF-1α as an agonist and its potency as an inhibitor of HIV-1 mediated cell-to-cell fusion, and verify that the amino-terminal methionine did not compromise the anti-HIV activity. Fusion was measured in a coculture system that utilizes a luciferase reporter system (Aguilar-Cordova *et al.*, 1994). Preliminary experiments (not shown) indicated that luciferase activity increased continuously over the first 8–10 hr of coculture and could be completely blocked with soluble CD4 if present within the first 2 hr of coculture. We tested SDF-1α for the ability to block fusion in this assay; the data are shown in Fig. 5A. The induction of luciferase activity was blocked by SDF-1α in a concentration-dependent manner. An IC_{50} value

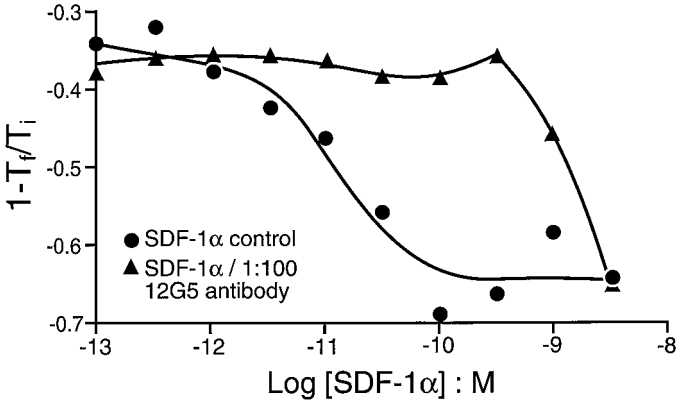

Fig. 4. Effect of 12G5 antibody on responses to SDF-1α. Control responses (●) and after 30-min incubation with 0.7× L-15/0.1% BSA containing 12G5 antibody (▲, 16.9 μg/ml).

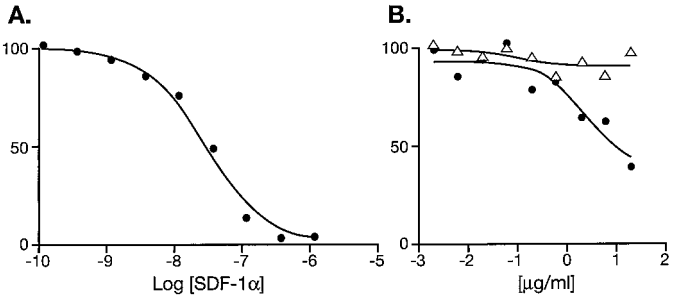

Fig. 5. Effect of SDF-1α (A) and 12G5 antibody (B) on viral fusion. Ordinate axes, percent of control cell fusion; abscissae, logarithms of molar concentrations of SDF-1α (A) and 12-G5 antibody (B) and 52R2 antibody as control (μg/ml).

of 23 ± 4 nM (n = 16) was calculated for SDF-α. Control experiments (not shown) indicated that SDF-1α had no direct inhibitory effect on luciferase gene expression or enzyme activity in cells that did not contain CXCR4.

The monoclonal antibody for CXCR4, 12G5, shown to be an antagonist in the melanophore system, is also known to block HIV-1 mediated cell-to-cell fusion (McKnight *et al.*, 1997). This result was confirmed in these studies (Fig. 5B), in which the IC_{50} was found to be 3.5 μg/ml (±1.1 μg/ml, n = 2) when the antibody was preincubated with cells for 1 hr before coculture. An isotype control antibody did not inhibit fusion over a range of concentrations up to 10 μg/ml.

The involvement of seven transmembrane receptors, such as CXCR4 in HIV viral fusion, has broad implications for the treatment of this disease. The discovery that seven transmembrane receptors are required for HIV-1 entry into cells offers a pharmacologically and chemically tractable target. Thus, if interaction of CXCR4 with either CD4 and/or the viral envelope (through gp120) can be disrupted, then the sequential process initiated by the tripartite protein interaction may stop fusion of cells (and subsequent viral entry). This is indicated by the blockade of viral fusion by SDF-1α. It is presently unclear whether activation of CXCR4 is required for the process of viral fusion, but the data showing that viral fusion is blocked by the 12G5 antibody, a ligand that does not produce responses in the melanophore system, indicates that this is not a requirement. This behavior is similar to the studies on chemokine receptor 5 as a coreceptor for HIV-mediated fusion where indirect evidence for the lack of receptor activation (with respect to G-protein) was obtained with CCR5/CCR2b receptor chimeras and mutants (Edinger *et al.*, 1997; Farzan *et al.*, 1997). In these studies, mutant receptors that were unable to mediate physiological responses facilitated viral fusion nevertheless.

Although receptor activation does not seem to be required for inhibition of viral fusion, the relationship between ligand occupancy and receptor activation is unclear. Specifically, if ligand occupation of CXCR4 is required to block viral fusion, then the concentration of CXCR4 agonist ligand useful for protection against HIV-1 infection may be considerably greater than the ED_{50} for G_i protein activation. This is attributable to the well-known disparity between physiological responses and receptor occupancy brought on by agonist intrinsic efficacy and amplification of signals through stimulus-response mechanisms (i.e., effective receptor reserve). This is suggested by the 434-fold increase in concentration of SDF-1α (over the ED_{50} for melanophore response) required to

inhibit viral fusion. The fact that the dose-response curve to SDF-1α was shifted 100-fold to the right by 12G5 antibody indicates a considerable effective receptor reserve in this preparation for this ligand.

Under these circumstances, achieving a saturating receptor occupancy by SDF-1α may require considerably higher concentrations of SDF-1α than the pharmacologic ED_{50}. A further unknown in the comparison of the two systems is the unknown receptor densities on the two cell lines. This latter factor may further distance the concentrations required to activate G_i proteins and prevent cell fusion. Further studies are required to elucidate the relationship between G protein activation by CXCR-4 and the role of this receptor as an HIV coreceptor for viral entry.

The ability to monitor CXCR4 function still has broad implications for the screening of receptor active ligands and therapeutic approaches in this area. Presently, the extent to which the screening of CXCR4 function in a seven-transmembrane receptor system will assist in finding drugs to inhibit viral fusion is unknown. The theoretical basis of this approach is that the melanophore/G_i-protein matrix will function as a detection system for CXCR4 conformational states and that these will translate into differences in the interaction of the CXCR4 receptor and proteins of the HIV viral coat.

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