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The CXCR4 agonist ligand stromal derived factor-1 maintains high affinity for receptors in both G_{α_i}-coupled and uncoupled states

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Abstract

The α chemokine receptor CXCR4 and its only characterized chemokine ligand, stromal cell-derived factor-1 (SDF-1), are postulated to be important in the development of the B-cell arm of the immune system. In addition, CXCR4 is a critical coreceptor in support of viral entry by T-cell line tropic strains (X4) of the Human Immunodeficiency Virus Type 1 (HIV-1), viral variants which predominate in some infected individuals in end stage disease. SDF-1 can block X4-tropic HIV-1 infection of CD4 + target cells in vitro, and allelic variants of the human gene encoding SDF-1 in vivo correlate with delayed disease progression. Therefore, CXCR4 may be an appropriate target for therapeutic intervention in acquired immunodeficiency syndrome (AIDS), and knowledge of the pharmacology of SDF-1 binding to its cognate receptor will be important in the interpretation of these experiments. We report here a K_d derived using a competition binding assay of 4.5 nM for CXCR4 endogenously expressed on peripheral blood monocytes and T-cells. This affinity is similar to that which SDF-1 exhibits when binding to endogenous CXCR4 on an established immortal Jurkat T-cell line as well as recombinant CXCR4 transfected into Chinese Hamster Ovary (CHO) cells. We also demonstrate that the determined affinity of SDF-1 for CXCR4 is reflective of its ability to induce a CXCR4-mediated signal transduction in these different cell types. Furthermore, using *Bordetella pertussis* toxin, we observe that high affinity binding of SDF-1 to CXCR4 is independent of the G-protein coupled state of the receptor, as uncoupling of G-protein did not lead to the appearance of measurable low affinity SDF-1 binding sites. Moreover, binding affinity and receptor number were unaffected by uncoupling for both recombinant and endogenously expressed CXCR4. Thus, SDF-1 is novel among agonist ligands of G protein-coupled receptors in that it appears to have equal affinity for both the G protein-coupled and uncoupled states of CXCR4.

Keywords: Chemokine; G-protein coupled receptor; Receptor coupling; HIV (human immunodeficiency virus)

1. Introduction

Chemokines are small (8–10 kDa) polypeptides initially characterized by their role as mediators of inflammatory responses. It is now widely accepted that these secreted proteins play a role in the normal physiology of the immune system, as well as in orchestrating leukocyte recruitment and activation in the context of inflammatory and infectious diseases (reviewed in Baggiolini, 1998; Baggiolini et al., 1997; Luster, 1998; Teran and Davies, 1996). The majority of chemokines belong to one of two major subfamilies—the β or CC chemokines in which two conserved cysteines in the amino terminus of these proteins are adjacent to each other, and the α or CXC chemokines in which these two cysteines are separated by an intervening residue. Receptors for the chemokines are members of the super-family of G-protein coupled receptors, characterized by seven transmembrane spanning regions, which couple to heterotrimeric G-proteins (Murphy, 1994; Premack and Schall, 1996). To date, genes encoding over 50 chemokine ligands and at least 10 CC receptors and five CXC receptors have been cloned and characterized. There is considerable promiscuity within this system, i.e. a particular receptor may bind more than one chemokine and a particular chemokine may bind to more than one

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receptor. However, the temporal and spatial regulation of chemokine expression in vivo suggests that there is much more specificity inherent to this system than would first appear.

The CXC chemokine stromal cell-derived factor 1 (SDF-1) is the only known chemokine able to bind to and activate CXCR4 (Nagasawa et al., 1994; Tashiro et al., 1993). SDF-1 stimulates rapid receptor-mediated intracellular calcium mobilization and signaling through a pertussis toxin sensitive G_i-coupled pathway (Moepps et al., 1997). In addition, SDF-1 induces rapid endocytotic CXCR4 internalization (Amara et al., 1997; Forster et al., 1998; Signoret et al., 1997; Tarasova et al., 1998) and the efficiency of receptor cycling back to the cell surface following internalization varies with cell type (Tarasova et al., 1998). Different downstream pathways, both receptor phosphorylation dependent and independent, appear to differentially regulate SDF-1-mediated signaling and internalization as a mutant CXCR4 receptor with a truncated carboxy-terminus demonstrates deficiencies in chemokine-triggered receptor phosphorylation, internalization and desensitization, but not calcium mobilization (Haribabu et al., 1997).

Mice lacking either SDF-1 or CXCR4 die perinatally and exhibit defects in vascular development, neuronal development, hematopoiesis and cardiogenesis that are identical in both knockouts (Ma et al., 1998; Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). This striking similarity between the phenotype of the two knockouts gives credence to the monogamous relationship between SDF-1 and CXCR4. Response to SDF-1 and expression of CXCR4 occurs at a very early stage of embryonic development and appears to be widely utilized whenever cell migration is required (McGrath et al., 1999).

Chemokine receptors are also obligatory participants in the entry of HIV-1 into host cells, with preferential interaction of viral envelope glycoproteins with different chemokine receptors underlying the cellular tropism exhibited by different viral strains. In this regard CXCR4, which is expressed on peripheral blood lymphocytes (Bleul et al., 1997; Loetscher et al., 1994), endothelial cells (Gupta et al., 1998; Volin et al., 1998), microglial neuronal cells (Lavi et al., 1997), and hematopoietic progenitor cells (Deichmann et al., 1997), facilitates entry of syncytium-inducing or T-tropic (T-cell line tropic, now X4-tropic) strains into $CD4^+$ cells (Feng et al., 1996). CCR5, a receptor expressed preferentially on activated T cells (Berkowitz et al., 1998; Bleul et al., 1997) and differentiated macrophages (Di Marzio et al., 1998; Naif et al., 1998), is involved in entry of non-syncytium inducing (NSI or M-tropic, now R5 tropic) HIV-1 strains (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996).

In vitro, the interaction of SDF-1 with CXCR4 serves as a potent block to cellular infection by X4-tropic strains of HIV-1 (Bleul et al., 1996; Oberlin et al., 1996). Moreover, these antiviral effects of SDF-1 are reduced when the carboxy-terminally truncated variant of CXCR4 is the HIV-1 coreceptor, indicating that a portion of the inhibitory activity of SDF-1 may arise from its ability to rapidly clear CXCR4 from the cell surface (Amara et al., 1997). The enhanced antiviral activity of an N-terminally modified recombinant form of SDF-1 (met-SDF-1), which prolongs CXCR4 down modulation as compared to native SDF-1 (Yang et al., 1999), supports this hypothesis. The characterization of a human polymorphism in the gene encoding SDF-1 that correlates with delayed progression to frank acquired immunodeficiency syndrome (AIDS) in HIV-1 infected individuals suggests this mechanism for entry may be physiologically important (Winkler et al., 1998). This mutation is postulated to prolong SDF-1 mRNA half-life and hence indirectly influence protein levels and receptor internalization. While these data suggest that a small molecule SDF-1 agonist mimetic may have therapeutic potential for the treatment of end stage AIDS when X4 tropic viruses tend to predominate (Koot et al., 1999; Tersmette et al., 1989a, b), SDF-1 also has the ability to potentiate M-tropic viral infection through a process that requires signaling through CXCR4 (Marechal et al., 1999). These data suggest that it may be preferable to block X4 viral entry with the use of CXCR4 antagonists rather than agonists.

Interpretation of the above data requires understanding and characterization of the interaction of SDF-1 with CXCR4. Our results indicate that the binding affinity of radiolabeled SDF-1 to endogenously and recombinantly expressed CXCR4 in all cells examined is identical and correlates to its functional potency in several signal transduction pathways. The uncoupling of G-protein from CXCR4 does not abrogate the ability of SDF-1 to bind to the receptor with high affinity nor does it affect the total number of high affinity receptor binding sites seen by SDF-1. These data suggest that SDF-1, unlike many other G protein-coupled receptor agonists, has the same affinity for CXCR4 in both the coupled and uncoupled states.

2. Experimental procedures

2.1. Materials

Radiolabeled SDF-1α (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Unlabeled SDF-1α and other α or β chemokines were purchased from Peprotech (Rocky Hill, NJ) unless otherwise noted. CHO cells stably expressing human CCR5 have been described (Siciliano et al., 1999). Jurkat cells were obtained from Aurora Biosciences (San Diego, CA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine and antibiotics (Life Technologies, Gathersburg, MD). Pertussis toxin and GTPγS were obtained from Calbiochem (San Diego, CA), Gpp(NH)p and protease inhibitors were obtained from Sigma (St.

Louis, MO) Anti-CXCR4 antibody (12G5) was obtained from Pharmingen (San Diego, CA). Plasmapheresed leukocytes were obtained from the University of Pennsylvania blood center.

2.2. Cloning and stable expression of CXCR4

The cDNA encoding CXCR4 was cloned by PCR using primers based upon the published human sequence (Loetscher et al., 1994) using lymph node cDNA (Clontech, Palo Alto, CA) as a template. The sequence of the coding region was verified by dideoxy sequencing, excised from pBluescript using *Hin*dIII and *Not*I, and then ligated into the mammalian expression vector pBJ-neo (Daugherty et al., 1996). For stable expression of CXCR4, 10^6 CHO dhfr$^-$ cells (CCL-61; ATCC, Rockville, MD) were transfected with 20 μg of DNA using a standard calcium phosphate procedure (Specialty Media, Lavallette, NJ). Six hours post-transfection, cells were re-fed with selection media (Hams F-12 supplemented with antibiotics and L-glutamine, 10% fetal bovine serum, 1 mg/ml Geneticin; Life Technologies). After 14 days, surviving cells were pooled and cloned by limiting cell dilution into 96-well microtiter plates. Wells containing a single focus were expanded and individual clones were selected on the basis of SDF-1α binding and fluorescence activated cell sorting using anti-CXCR4 antibody. Clonal cell lines were then maintained in selection media.

2.3. Preparation of CXCR CHO cell membranes

Plasma membranes from CXCR4 expressing CHO cells were prepared as described (Siciliano et al., 1990). Briefly, attached cells were washed twice with calcium/magnesium free phosphate-buffered saline (PBS, Life Technologies), harvested using enzyme-free dissociation media (Specialty Media) and pelleted by centrifugation at $1000 \times g$. The cell pellet was then resuspended in 1 mM Tris, pH 7.4 containing protease inhibitors (10 nM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, 5 μg/ml aprotinin, and 10 nM phosphoramidon). Cell membranes were disrupted by using a Teflon cell homogenizer and large debris pelleted by spinning at $1500 \times g$. Cell membranes were then pelleted by centrifuging the supernatant from the low speed spin at $20{,}000 \times g$. The membrane pellet was then resuspended in PBS and particulates dissociated by passing several times through a 1-cm^3 syringe equipped with a 27-gauge needle. Protein concentration of cell membrane preparations were determined by using a Bradford kit (BioRad, Richmond, CA) and aliquots were then either used in a binding assay or stored at −70°C until needed.

2.4. Preparation of primary monocytes and T-cells

Monocytic cells were purified by adherence to a plastic substrate as described (Weng et al., 1998), followed by incubation in 100 ng/ml macrophage colony stimulating factor (Biosource International, Camarillo, CA) for 24 to 48 h in RPMI 1640 containing 10% fetal calf serum (Life Technologies). Monocytic cells were then harvested using enzyme free-dissociation media and assayed for SDF-1α binding as described below. T-cells were purified from the mononuclear cell preparation by E-rosetting (Weng et al., 1998) followed by overnight incubation at 37°C. Cells were harvested by centrifugation, washed with binding buffer and assayed for SDF-1 binding. For pertussis toxin pretreatment, monocytes or T-cells were incubated with 100 ng/ml pertussis toxin for 18 h at 37°C.

2.5. Cyclic AMP accumulation assay

Twenty-four hours prior to assay, CHO CXCR4 cells were plated into a 24-well plate at a density of 100,000 cells per well. For pertussis treated cells, 100 ng/ml toxin was added immediately after plating and incubated for 24 h. At the time of assay, the media was aspirated and the cells were washed twice with calcium/magnesium free PBS. The cells were then overlaid with 200 μl assay media (DME, 0.5% bovine serum albumin; Life Technologies), containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) and incubated with shaking at 37°C. After incubating for 30 min, 50 μl of assay media (basal controls), or assay media containing 50 μM forskolin (10 μM final concentration; Sigma) or forskolin plus 1 μM to 4 pM SDF-1α was added. The cells were incubated for 5 min at 37°C, after which time the plates were placed on ice and the media aspirated to remove extracellular cyclic AMP (cAMP). 250 μl of 0.1 N NaOH was added to each well and the lysates were quantified for cAMP using an Amersham cAMP kit (RPA 509). The functional assay in Jurkat cells was performed as follows: 48 h prior to assay, the cells were switched to growth media containing 0.5% fetal calf serum. For pertussis toxin treatment, 50 ng/ml was added to the cells 24 h prior to assay. For the assay, the cells were pelleted at $1000 \times g$, washed once with calcium/magnesium-free PBS, and resuspended in a 96-deep well box at 500,000 cells per assay point. Intracellular cAMP accumulation was then measured as described for CHO CXCR4 cells except that the assay was terminated by the addition of 1 ml of 4°C PBS followed by centrifugation at $1000 \times g$. The media/PBS mixture was aspirated and 250 μl of 0.1 N NaOH was added to each pellet. cAMP was then quantified as described above. Functional assays using primary monocytes with or without pertussis toxin treatment (50 ng/ml added 24 h prior to assay) was performed as described for Jurkat cells except that 750,000 cells per point were assayed.

2.6. CXCR4 binding assays

Binding of SDF-1α to CXCR4 was measured in both whole cells and membranes. 5×10^4 CXCR4 expressing CHO cells, 2.5×10^5 Jurkat cells, 7.5×10^5 primary monocytes or 2.5×10^6 primary T-cells were incubated

with approximately 50 pM of ^{125}I-SDF-1α and unlabeled SDF-1α at concentrations ranging from 1 μM to 4 pM. Final assay volume was 250 μl in a binding buffer of phosphate-buffered saline (Mg^{2+}/Ca^{2+} free) pH 7.2, 5 mM EDTA (Sigma), and 0.25% bovine serum albumin (Sigma). For binding to CXCR4 CHO membranes, 5 μg of membranes in HEPES binding buffer (50 mM HEPES, pH 7.4; Calbiochem; 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25% bovine serum albumin) was used in a final assay volume of 150 μl. After incubating for 1 h at room temperature with shaking, the reaction was terminated by filtering through a 0.1% polyethylenimine (Sigma) soaked GF-C filter plate (Packard) using a Packard Filtermate cell harvester and the plate washed with approximately 750 μl of 50 mM HEPES (Sigma), pH 7.4, 500 mM NaCl chilled to 4°C. The plates were dried, scintillant added and counted on a Packard TopCount. Binding assays with primary T-cells were harvested using a Tomtec harvester and 0.1% polyethylenimine soaked GF-C filter paper, washed with approximately 2 ml of chilled HEPES wash buffer and counted in a gamma counter. Non-specific binding was measured in the presence of 1 μM SDF-1α. Binding results were analyzed using Microsoft Excel and GraphPad Prism software.

2.7. Treatment of CXCR4 membranes with non-hydrolyzable GTP analogs

Five micrograms of CXCR4 CHO membranes were preincubated with various concentrations of GTPγS or Gpp(NH)p ranging from 0.3 nM to 3 μM for 30 min in HEPES binding buffer. Following pretreatment, unlabeled SDF-1α and 50 pM of [^{125}I]SDF-1α were added to a final assay volume of 150 μl. The membranes were then incubated for an additional 60 min at room temperature. The reaction was terminated and samples processed as described above for the ligand binding assay.

2.8. Microphysiometer

Functional assay using microphysiometry was performed as described (Weng et al., 1998). Briefly, CHO cells stably expressing human CXCR4 were plated onto Transwell cell capsule cups (Molecular Devices, Sunnyvale, CA) at a density of 3.3×10^5 cells/ml/cup in Ham's F-12 medium plus 10% fetal bovine serum (Life Technologies). The cups were allowed to incubate overnight and then transferred to the microphysiometer sensor chambers (Cytosensor, Molecular Devices). Cells were then allowed to equilibrate in running medium (RPMI 1640 plus 0.1% bovine serum albumin, Molecular Devices) for 2 h to obtain a stable acidification rate. Once a stable acidification rate was obtained, cells were exposed to several concentrations of SDF-1α made in running medium for 6 min. Acidification rates were then measured at 2 min intervals using a flow rate of 100 μl/min.

2.9. Calcium flux

Calcium flux was measured using purified T-cells or monocytes labeled with Fluo-3 acetoxymethyl ester (Molecular Probes, Eugene OR) as previously described (DeMartino et al., 1994) using 4×10^5 monocytes or 1×10^6 T-cells per measurement point. Maximal calcium flux was determined for each concentration of SDF-1α

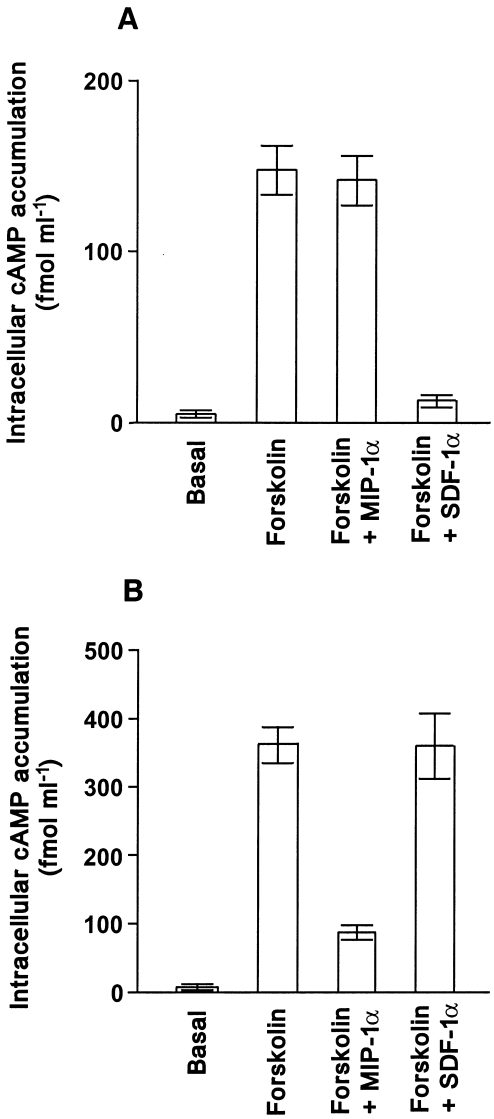

Fig. 1. Inhibition of forskolin-stimulated cAMP accumulation by SDF-1α in CHO CXCR4 (Panel A) and by Mip1α in CHO CCR5 cells (Panel B). CXCR4 or CCR5 expressing CHO cells were incubated for 30 min with 3-isobutyl-1-methylxanthine and then treated with 10 μM forskolin for 5 min at 37°C to induce adenylyl cyclase activity and subsequent cAMP accumulation in the presence or absence of 38 nM MIP-1α or 50 nM SDF-1α. Following incubation with forskolin with or without chemokine, cells were lysed and intracellular cAMP measured using Amersham kit RPA 509 as described in Section 2. Basal activity was measured in the absence of forskolin pretreatment. Stimulation with MIP-1α or SDF-1α in the absence of forskolin treatment had no effect on basal levels of cAMP accumulation (data not shown). Data presented are the mean ± S.E.M. for three assays with each condition performed in triplicate.

and an EC_{50} determined by fitting the data to a sigmoidal dose response curve using GraphPad Prism software.

3. Results

3.1. SDF-1α inhibits forskolin-stimulated cAMP accumulation in CXCR4 expressing CHO cells and the inhibition is sensitive to pertussis toxin treatment

Signaling by SDF-1α in a stable cell line expressing CXCR4 was determined by measuring the ability of SDF-1α to inhibit forskolin stimulated cAMP accumulation, the classical signal transduction pathway associated with G_i-coupled receptors (Birnbaumer, 1990). SDF-1α addition completely inhibited forskolin-stimulated cAMP accumulation in CHO CXCR4-transfected (Fig. 1A) but not CHO CCR5-transfected cells (Fig. 1B), confirming that SDF-1α-induced inhibition is mediated through CXCR4. Reciprocally, MIP-1α, a CCR5 ligand, inhibited forskolin-stimulated cAMP accumulation in CHO cells transfected with CCR5 (Fig. 1B), which is also coupled through G_i, but had no effect on CXCR4-transfected cells (Fig. 1A). We further characterized this effect by titrating the amount of SDF-1α required for inhibition and determined an EC_{50} of 0.36 ± 0.1 nM ($n = 4$) (Fig. 2A). As was expected, pertussis toxin pretreatment of the CHO CXCR4 cells completely abolished the ability of SDF-1α to inhibit forskolin-stimulated cAMP accumulation (Fig. 2B).

3.2. High affinity binding of SDF-1α to CXCR4 is not affected by pertussis toxin treatment in recombinant CXCR4 CHO cells

The ability of SDF-1α to bind with high affinity to CXCR4 has been documented (Hesselgesser et al., 1998; Ueda et al., 1997), but little is known about the ability of SDF-1α to interact with the uncoupled state of the receptor. To address this question, CHO CXCR4 expressing cells were treated overnight with pertussis toxin to effectively uncouple all the receptors. Surprisingly, pertussis toxin did not affect high affinity binding of SDF-1α (Fig. 3A). Scatchard analysis of the binding data (Fig. 3B) indicated no difference in the observed affinity ($K_d = 4.0 \pm 2.4$ nM, for untreated vs. 4.8 ± 1.9 nM, for pertussis toxin pretreated cells; $n = 4$). Furthermore, pertussis toxin pretreatment did not effect total number of binding sites per cell ($1.2 \pm 0.2 \times 10^6$ $n = 4$ for untreated vs. $1.0 \pm 0.2 \times 10^6$ $n = 4$ for pertussis toxin treated) and a single high affinity binding site was observed for both the treated and untreated cells (Hill coefficient ~ 1). To extend these observations, the effects of the non-hydrolyzable GTP analogs GTPγS or Gpp(NH)p on the binding of [^{125}I]SDF-1α to membranes prepared from CHO CXCR4 cells was examined. The presence of GTPγS or Gpp(NH)p at concentrations from 0.3 nM to 3 μM did not significantly

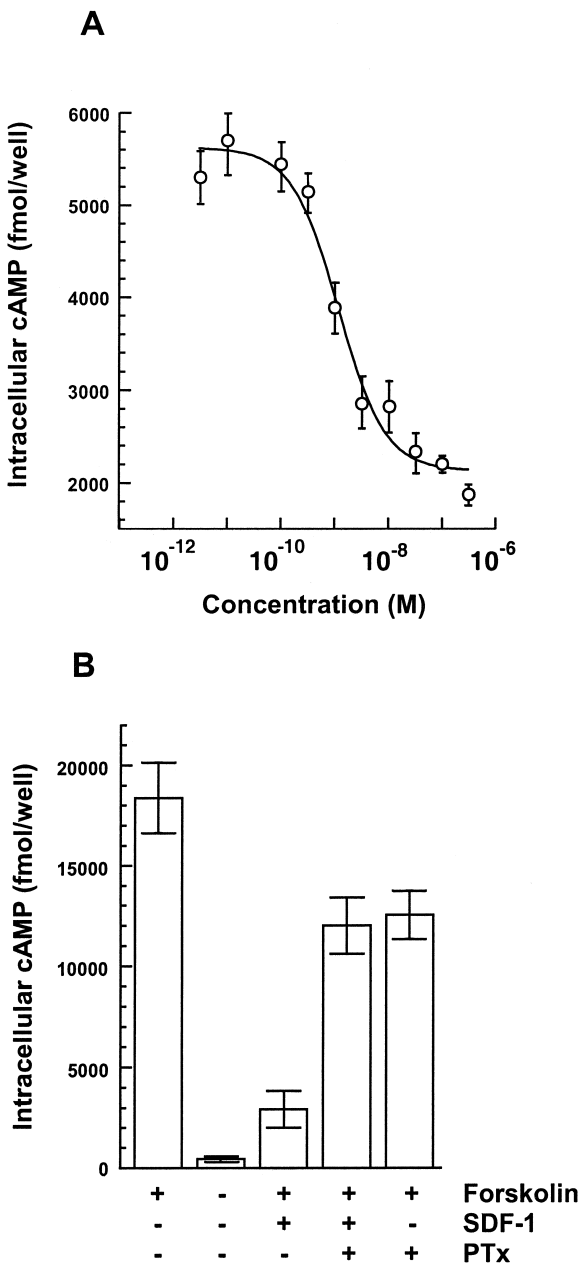

Fig. 2. Inhibition of forskolin stimulated cAMP accumulation in CHO CXCR4 cells by pertussis toxin. Panel A: inhibition of forskolin-stimulated cAMP levels by increasing concentrations of SDF-1α. 1×10^5 CHO CXCR4 cells were pretreated for 30 min with 10 μM forskolin followed by the addition of SDF-1α at various concentrations ranging from 500 nM to 5 pM. Cells were incubated for an additional 5 min with ligand and cAMP levels determined as described. Effect at each concentration of SDF-1α was measured in triplicate and the results were fitted to a standard one-site competition curve. The EC_{50} was 0.36 nM with a maximal effect typically observed at 50 to 100 nM SDF-1α. Data represent mean ± S.D. Panel B: CHO CXCR4 cells were pretreated with 100 ng/ml pertussis toxin 24 h prior to assay and the effect on cAMP accumulation was compared to untreated cells. Cells were stimulated for 30 min with 10 μM forskolin prior to addition of 50 nM SDF-1α. Following a 5-min incubation with chemokine, intracellular cAMP was measured as described in Panel A. Basal cAMP levels were measured in the absence of any forskolin stimulation and were not significantly affected by pertussis toxin pretreatment (data not shown). Data represent mean ± S.D.

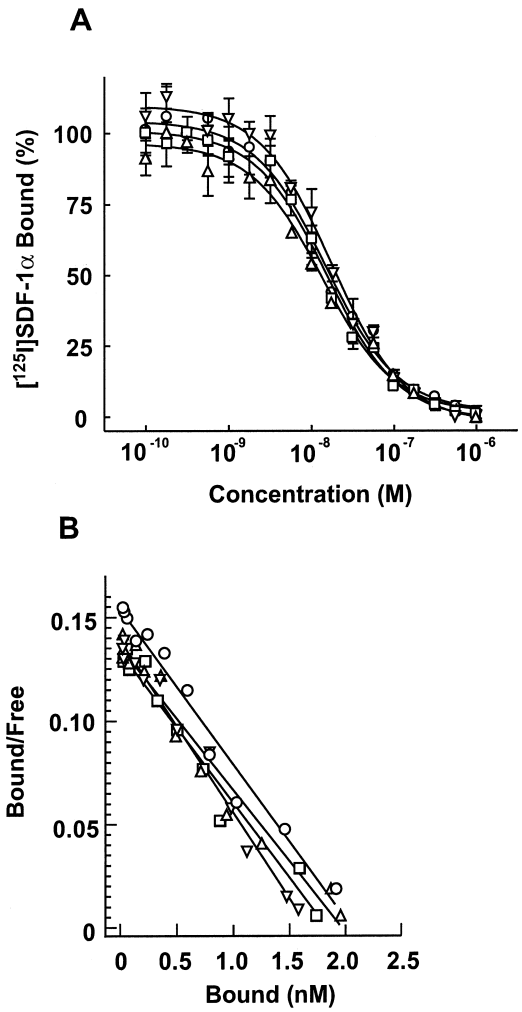

Fig. 3. Pertussis toxin pretreatment does not effect binding of [^{125}I]SDF-1α to recombinant CHO CXCR4 cells. CHO cells expressing CXCR4 were treated with PTX for 24 h with 300 ng/ml (□), 30 ng/ml (△) or 3 ng/ml (▽) and compared to untreated controls (○) for their ability to bind [^{125}I]SDF-1α in a cold competition assay. Panel A: cold competition binding assay using 50,000 cells and 50 pM [^{125}I]SDF-1α per assay point. Following a 60-min incubation at room temperature, the assay was terminated via filtration and results analyzed as described in Section 2. Data represent mean ± S.D. of quadruplicate determinations from a single representative experiment that was repeated four times with identical results. Panel B: Scatchard transformation of binding curves from Panel A (symbols are as above).

inhibit [^{125}I]SDF-1α binding (Fig. 4A). The observed K_d for SDF-1α was 7.6 ± 3.4 nM ($n = 4$), 5.4 ± 3.2 nM ($n = 4$) or 7.7 ± 1.7 nM ($n = 4$) in control membranes and membranes pretreated with 500 nM GTPγS or Gpp(NH)p, respectively. These data are consistent with the inability of pertussis toxin to affect SDF-1α binding in CHO CXCR4 cells.

3.3. Microphysiometer analysis of SDF-1α-induced signaling in CHO CXCR4 expressing cells

Because we found it surprising that SDF-1α bound with the same affinity to the coupled and uncoupled state of CXCR4, we confirmed that pertussis toxin treatment abrogated SDF-1α-mediated signaling in these cells using a microphysiometer. The microphysiometer measures the rate of agonist-induced media acidification and, therefore, reflects the sum of all cellular signaling. SDF-1α stimulated a rapid and transient increase in the extracellular acidification rate in CHO CXCR4 cells (Fig. 5, open symbols). The EC_{50} of approximately 5 nM is consistent with the binding K_d in these cells but is approximately 10-fold less potent

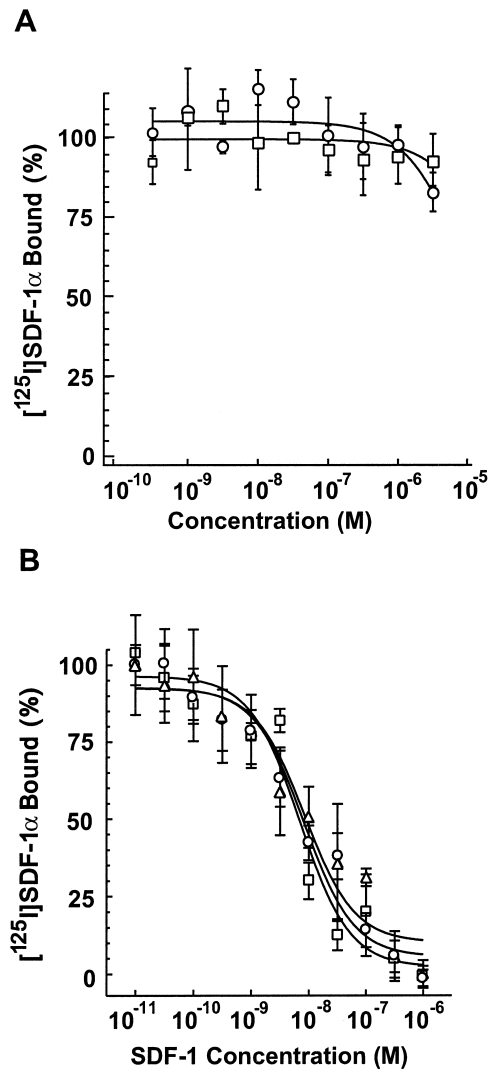

Fig. 4. Effect of GTPγS and Gpp(NH)p on high affinity binding of SDF-1α to CXCR4 in membranes. Panel A: freshly prepared CHO CXCR4 membranes (5 μg per point) were preincubated with increasing concentrations of GTPγS (○) or Gpp(NH)p (□) for 30 min at room temperature. Following preincubation, 50 pM [^{125}I]SDF-1α was added and the membranes incubated for an additional 60 min. The binding was then terminated by filtration and processed as described for the whole cell binding assay. Panel B: effect of toxin pretreatment on binding of SDF-1α to CXCR4 CHO membranes. CHO CXCR4 membranes (5 μg) were preincubated with 500 nM GTPγS (□), Gpp(NH)p (△) or buffer alone (○) for 30 min. Following preincubation, cold competition binding was performed using 1 μM to 0.05 nM cold SDF-1α and 50 pM [^{125}I]SDF-1α as described in Panel A. Data represent mean ± S.D. of triplicate determinations from a single representative experiment.

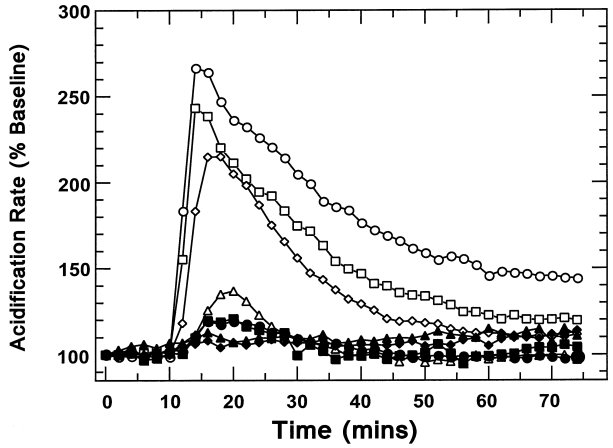

Fig. 5. Microphysiometric analysis of CXCR4 expressing CHO cells in response to SDF-1α stimulation. CXCR4 CHO cells untreated (open symbols) or pretreated 24 h prior to assay with 100 ng/ml pertussis toxin (closed symbols) were exposed to 300 nM (○, ●), 100 nM (□, ■), 10 nM (◇, ◆) or 1 nM (△, ▲) SDF-1α for 6 min. Acidification rates were then measured at 2-min intervals following SDF-1α exposure. No response was observed in untransfected CHO cells using 300 nM SDF-1α (results not shown). Data points represent a single measurement made at each time point from a single representative experiment. Microphysiometry was repeated three times with identical results.

than the EC_{50} measured in the cyclase assay. As expected, SDF-1α failed to affect the acidification rate in pertussis toxin pretreated cells (Fig. 5, filled symbols), confirming the role of G_i-mediated signaling in the induction of this response.

3.4. SDF-1α binding to endogenously expressed CXCR4 in a T-cell line and in primary leukocytes is identical to that in the recombinant cell line and is also insensitive to pertussis toxin pretreatment

CXCR4 is expressed in our recombinant CHO cell line at levels 10- to 30-fold greater than typically observed in cells endogenously expressing the receptor (Hesselgesser et al., 1998; Lee et al., 1999a,b). Effects of uncoupling on binding in overexpressed systems may not necessarily reflect that observed in vivo, so the effect of uncoupling on SDF-1α binding to CXCR4 was performed in a Jurkat T-cell line that endogenously expresses CXCR4. As was observed in CHO CXCR4 cells, pertussis toxin pretreatment of Jurkat T-cells not only did not affect the affinity of SDF-1α for CXCR4 (Fig. 6A), but the affinity of 3.1 ± 1.2 nM ($n = 4$) for untreated and 3.0 ± 1.0 nM ($n = 4$) for pertussis toxin treated Jurkat cells was nearly identical to that measured in our recombinant cell line. Also, as is true in the recombinant system, the number of binding sites determined by Scatchard analysis was not affected by pertussis toxin treatment (approximately 52,000 receptors per cell; Fig. 6A, insert). Previously, Hesselgesser et al. (1998) demonstrated that pertussis toxin treatment of Jurkat cells totally abolished an SDF-1α increase in extracellular acidification rate. We observed the same inhibition of signaling by pertussis toxin using the cAMP accumulation assay (Fig. 6B) and determined an

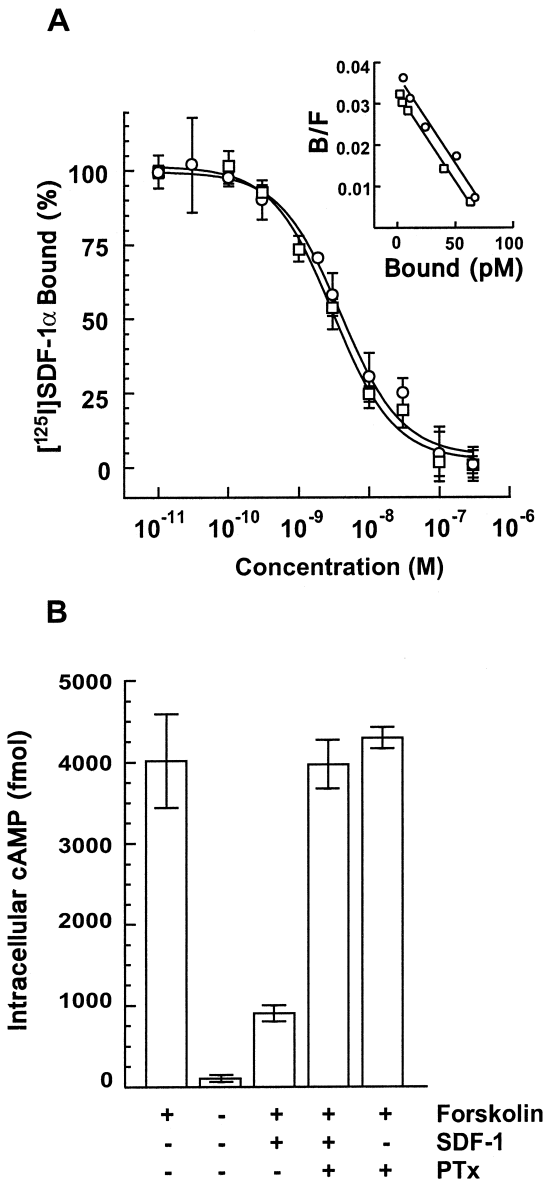

Fig. 6. Effect of pertussis toxin treatment on binding and functional activity of endogenously expressed CXCR4 in Jurkat T-cells. Panel A: effect of pertussis toxin on binding of SDF-1α to Jurkat cells. Cold competition binding assays were performed using Jurkat cells pretreated for 24 h with 100 ng/ml pertussis toxin (□) and compared to untreated cells (○). 2.5×10^5 cells and 50 pM [^{125}I]SDF-1α were used per assay point. Assays were run in triplicate and the results are from a representative titration curve. Panel A (insert): Scatchard analysis of binding data comparing untreated (○) versus pertussis toxin (□) treated Jurkat cells. K_d and maximal binding were not significantly different in the treated versus untreated cells. Panel B: cAMP accumulation assay. Jurkat cells were pretreated with 100 ng/ml pertussis toxin 24 h prior to assay and the effect on cAMP accumulation was compared to untreated cells. Cells were stimulated for 5 min with 10 μM forskolin in the presence or absence of 30 nM SDF-1α. Following the incubation, intracellular cAMP was measured as described in Section 2. Basal activity was measured in the absence of forskolin stimulation. Data are mean ± standard deviation of triplicate determinations from a single representative experiment.

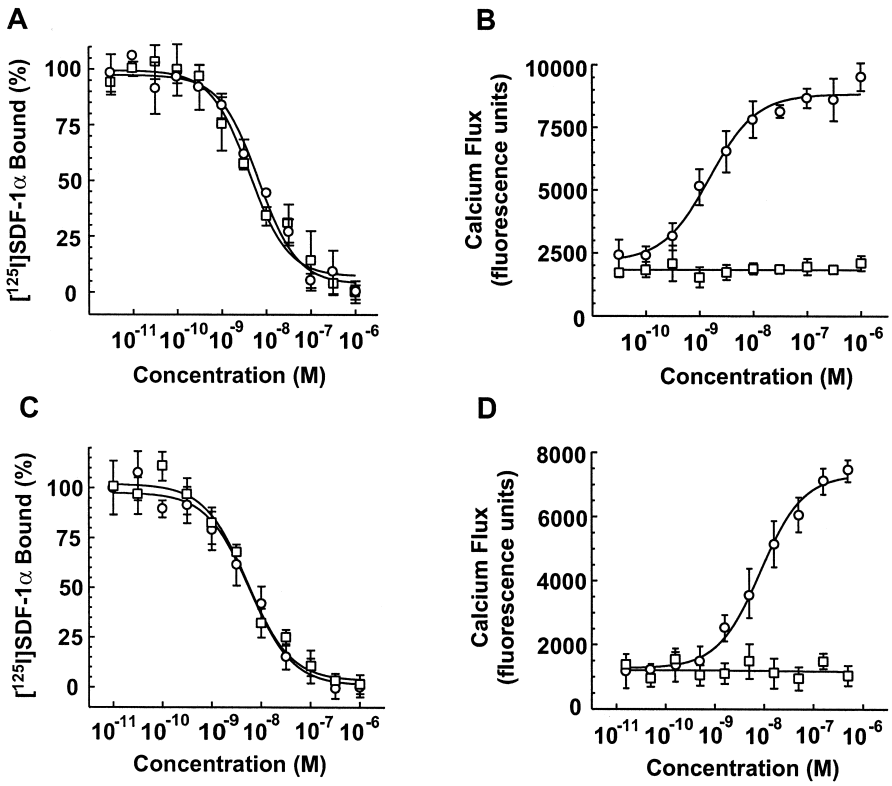

Fig. 7. Effect of pertussis toxin treatment on binding and functional activity in peripheral blood monocytes and T-cells. Panels A,C: competition binding of [^{125}I]SDF-1α to peripheral blood monocytes (Panel A) or T-cells (Panel C). For the binding assay, 7.5×10^5 monocytes or 2.5×10^6 T-cells were incubated with 50 pM [^{125}I]SDF-1α and increasing amounts of unlabeled SDF-1α. Binding to untreated (○) or 100 ng/ml pertussis toxin pretreated (□) cells was identical. Data are mean ± standard deviation of quadruplicate determinations from a single representative experiment. Panels B,D: SDF-1α mediated CXCR4-dependent calcium flux measured in primary monocytes (Panel B) or T-cells (Panel D). Time-based calcium flux using 4×10^5 monocytes or 1×10^6 T-cells was measured with increasing amounts of SDF-1α (○). Each point represents the maximal flux induced at the given SDF-1α concentration and is an average of three determinations. Pretreatment of primary cells with 100 ng/ml pertussis toxin (□) completely eliminates SDF-1α induced calcium flux. Binding and functionality were measured concurrently from the same batch of primary cells. Data represent mean ± S.D. from a single representative experiment.

EC_{50} of approximately 3 nM for SDF-1α agonism using this assay (data not shown).

To determine the in vivo relevance of the CXCR4 pharmacology observed in the above cell lines, SDF-1α binding and signaling were examined in primary human monocytes and T-cells. As was observed in the recombinant CXCR4 CHO cell line and the Jurkat T-cell line, the uncoupling of CXCR4 had no effect on the binding affinity for SDF-1α in either primary cell (Fig. 7A and C). Furthermore, the SDF-1α binding affinity of 5.4 ± 1.7 nM ($n = 4$) for untreated and 4.2 ± 1.2 nM ($n = 4$) for pertussis toxin treated for monocytes, and 4.7 ± 1.6 nM ($n = 3$) for untreated and 4.8 ± 0.6 nM ($n = 3$) for pertussis toxin treated T-cells was nearly identical to SDF-1α affinities measured in the CHO CXCR4 and Jurkat cell lines. Maximal SDF-1α binding was not significantly different in the coupled or uncoupled states for both primary cell types. To confirm that toxin treatment inhibited SDF-1α-induced signaling via CXCR4, concurrent functional assays were run on the primary cells (Fig. 7B and D). While cAMP accumulation measurements provided a robust means of measuring functionality in our recombinant cell line and the Jurkat T-cells, calcium flux measurements proved to be the more reproducible indicator of functionality in primary cells given their limited number and lower receptor expression levels (unpublished observation). An SDF-1α dose-dependent increase in calcium flux was measured in both primary monocytes ($EC_{50} = 4.3 \pm 2.5$ nM, $n = 4$) and T-cells ($EC_{50} = 6.4 \pm 2.6$ nM, $n = 3$) and, as expected, pertussis toxin treatment totally abolished this signaling in both cell types (Fig. 7B and D). Interestingly, the EC_{50} as measured by calcium flux is identical to K_d as measured by SDF-1α binding in both monocytes and T-cells, although the calcium response and maximal SDF-1α binding in monocytes were greater than that in T-cells.

4. Discussion

We have shown that SDF-1, the only known ligand for CXCR4, binds with high affinity independent of the G-protein coupled state of the receptor on a stably expressing CXCR4 CHO cell line, on a T-cell line and in primary human leukocytes (results summarized in Table 1). Both

Table 1
Summary of binding affinity, functional activity and receptor number for recombinantly and endogenously expressed CXCR4

Cell line	Binding K_d (nM)		Functional EC_{50} (nM)	Receptors per cell ($\times 10^4$)	
	− pertussis toxin	+ pertussis toxin		− pertussis toxin	+ pertussis toxin
CHO CXCR4	4.0 ± 2.4 (4)	4.8 ± 2.4 (3)	0.36 ± 0.1(4)	120 ± 20 (4)	100 ± 20 (4)
Jurkat	3.1 ± 1.2 (4)	3.0 ± 1.0 (4)	3.0 ± 1.9 (3)	5.2 ± 1 (4)	4.8 ± 1.1 (4)
Primary monocyte	5.4 ± 1.7 (4)	4.2 ± 1.2 (4)	4.3 ± 2.5 (4)	ND	ND
Primary T-cell	4.7 ± 1.6 (4)	4.8 ± 1.6 (4)	6.4 ± 2.6 (3)	ND	ND

Binding affinity, functional activity and receptor number per cell for CXCR4 recombinantly expressed in CHO cells (CHO CXCR4) or endogenously expressed in a Jurkat T-cell line, primary monocytes and primary T-cells. SDF-1 functional EC_{50} was determined by measuring inhibition of forskolin stimulated cyclase activity (CHO/CXCR and Jurkat) or stimulation of calcium flux (primary monocytes and T-cells). Average receptor number for primary monocytes and T-cells was not determined (ND). Data is average value ± S.D. of indicated number of independent measurements (in parenthesis).

high affinity binding and absolute number of SD1-1α binding sites were unaffected in pertussis toxin treated cells relative to untreated cells, whereas the former did not signal as measured by forskolin-stimulated cAMP accumulation, calcium flux or microphysiometry. The lack of effect on receptor number confirms the absence of any measurable low affinity binding sites in the uncoupled state. Had uncoupling resulted in the appearance of a low affinity site below the limit of detection, then GTPγS or pertussis toxin would appear to cause a concentration-dependent decrease in the number of high affinity binding sites, as is observed with agonist ligands for other G protein-coupled receptors (Siciliano et al., 1990; Cascieri et al., 1996). We likewise observed no change in high affinity binding as a result of uncoupling CXCR4 in both primary monocytes and T-cells, confirming that the observations made the CHO CXCR4 cells accurately reflect CXCR4 pharmacology in primary cells. Several CXCR4 mutants have been described (Doranz et al., 1999) that bind SDF-1 but do not signal, although the reported affinities for the mutants and wild type receptor were 10–20-fold lower than determined in the present study. Our observations confirm this separation of the ability of SDF-1 to bind CXCR4 and to induce a signal and extend it to show that high affinity binding of SDF-1 to CXCR4 is identical in the coupled and uncoupled states.

A broad range of affinities of SDF-1 for CXCR4 have been reported by a number of investigators (Doranz et al., 1999; Haribabu et al., 1997; Hesselgesser et al., 1997, 1998; Ueda et al., 1997), but here we show a consistent K_d of 4–5 nM for both recombinantly expressed and endogenous CXCR4 in the cell types examined. We also confirmed (data not shown) earlier work demonstrating no difference in binding affinity between the two alternatively spliced forms of SDF-1 (Crump et al., 1997). Additionally, the affinity of SDF-1α for membranes prepared from CHO CXCR4 cells was nearly identical to that in whole cells. With regard to signal transduction, we determined that the functional potency of SDF-1α is nearly identical to its binding affinity as measured by quantitative calcium flux in primary cells, extracellular acidification rate in CHO CXCR4 cells or forskolin-stimulated cAMP accumulation in the Jurkat T-cell line. Interestingly, we found SDF-1α to be functionally 10-fold more potent in the recombinant cell line as measured by inhibition of forskolin-stimulated cAMP accumulation, which may be an artifact of receptor over-expression or G-protein coupling efficiency in the CHO cell heterologous expression system. This observation is consistent with data in the literature, for example in the β3 adrenergic receptor, where agonist potency (as measured by cyclase activation) increases with increasing levels of receptor expression (Whaley et al., 1994).

The binding of SDF-1 to CXCR4 induces a cascade of signaling events, which can be divided into events that are pertussis toxin sensitive and, thus, require G_i-mediated signaling and those that are pertussis toxin insensitive and mediate signaling via other transduction pathways. As we and others have shown, pertussis toxin pretreatment abolishes the inhibition of forskolin stimulated cAMP accumulation, calcium flux (Aiuti et al., 1997; Hesselgesser et al., 1998) and chemotaxis (Bleul et al., 1998; Vicente-Manzanares et al., 1998). SDF-1-induced internalization of CXCR4, which requires phosphorylation of residues in the CXCR4 C-terminus, appears to be the only major event insensitive to pertussis toxin treatment (Amara et al., 1997; Forster et al., 1998; Haribabu et al., 1997), although HIV T-tropic envelope-mediated cell fusion (KS, unpublished observations) and HIV infection (Amara et al., 1997) also do not appear to require G-protein mediated signaling through CXCR4. Given that HIV infection does not require signaling, the ability of SDF-1 to bind to both coupled and uncoupled CXR4 with high affinity would suggest that SDF-1 is a highly effective antiviral agent.

Although CXCR4 is widely expressed, there is increasing evidence that CXCR4 may be surface expressed in both a functional and non-functional state. During B-cell development, pre-B cells but not immature or mature B-cells have functional CXCR4, even though surface expression is nearly identical in both populations (Aiuti et al., 1997; Honczarenko et al., 1999). Regulation of CXCR4 function rather than expression may be the means by which B-cell compartmentalization is maintained. The ability of SDF-1 to bind and internalize non-functional receptor might allow the cell to quickly replace non-functional

receptor with signal competent receptor. In the case of B-cell lymphopoiesis, SDF-1-induced CXCR4 internalization does occur in cell populations that contain functional and non-functional CXCR4 at equivalent rates (Honczarenko et al., 1999). What is presently not known is the means by which CXCR4 functionality is regulated.

In the classical scheme for activation of G-protein coupled receptors, the activation and subsequent dissociation of G-proteins from the receptor is accepted as a component of the signal termination mechanism (Cascieri et al., 1996). This uncoupling of G-protein from the receptor generally leads to a receptor state with lower affinity for agonist. From receptor and ligand mutagenesis studies, it is apparent that chemokine binding and functionality can be separated, but the relationship of agonist binding affinity to coupling had not been previously explored. Given our observations that high affinity binding is equivalent in both the uncoupled and coupled states of CXCR4, SDF-1 could be defined as a "neutral agonist" given its lack of preference for the G-protein coupled or uncoupled state. While it is not known how unique this observation is among the other GPCRs, we have found that binding of radiolabeled MCP-1 to CCR2, and radiolabeled IP10 to CXCR3 is inhibited 60% and 70%, respectively, by non-hydrolyzable GTP analogs (unpublished observations), strongly suggesting that these agonists do have dramatically different affinities for the coupled and uncoupled states of these chemokine receptors. Similar observations have been made with other GPCRs whose ligands are peptides or proteins, including the receptors for substance P, C5a, calcitonin gene related peptide, vasoactive peptide, oxytocin and glucagon (Cascieri et al., 1992; Chatterjee and Fisher, 1995; Chicchi et al., 1997; Jasper et al., 1995; Shreeve et al., 2000; Siciliano et al., 1990). Thus, the paradigm that GPCR agonist affinity is regulated by G-protein coupling that was originally established using the biogenic amine receptors also generally holds true for the larger family of GPCRs, suggesting that the lack of an effect of coupling on SDF-1 binding affinity to the CXCR4 receptor is unique. However, other observations (Springer et al., manuscript in preparation) indicate that additional chemokines may also behave as "neutral agonists", and that this property may be of physiological relevance in the functioning of some of the members of this family.

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