cAMP Up-Regulates Cell Surface Expression of Lymphocyte CXCR4: Implications for Chemotaxis and HIV-1 Infection

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cAMP Up-Regulates Cell Surface Expression of Lymphocyte CXCR4: Implications for Chemotaxis and HIV-1 Infection

Steve W. Cole,* Beth D. Jamieson,* and Jerome A. Zack*†

The chemokine receptor CXCR4 mediates lymphocyte chemotaxis in response to stromal cell-derived factor-1 (SDF-1) and functions as a coreceptor for T cell-tropic strains of HIV-1. We examined the role of the cAMP-protein kinase A (PKA) signaling pathway in regulating expression of CXCR4. In response to exogenous dibutyryl cAMP or cAMP-inducing ligands, cell surface expression of CXCR4 was increased by up to 10-fold on CD3/CD28-stimulated PBMC and by up to sixfold on unstimulated PBMC. cAMP did not alter receptor mRNA levels or affect the size of the total CXCR4 pool. However, cAMP did significantly reduce CXCR4 internalization rates and thereby increased the fraction of the total CXCR4 pool expressed on the cell surface. cAMP-induced increases in CXCR4 expression counteracted SDF-1-induced receptor internalization and enhanced both chemotactic response to SDF-1 and cellular vulnerability to HIV-1 infection. Thus, altered chemokine receptor expression may provide one mechanism by which cAMP-inducing ligands influence lymphocyte localization and HIV pathogenesis. The Journal of Immunology, 1999, 162: 1392–1400.

Effective immune response requires the recruitment of functionally distinct leukocyte subsets to appropriate tissue sites (e.g., direction of hematopoietic progenitors and naive lymphocytes to lymphoid organs and mature lymphocytes to peripheral sites of inflammation). This recruitment process is believed to be mediated in part by chemokines, soluble messenger molecules secreted by target tissue cells to signal their status as lymphoid organs or inflamed tissue (1–3). Differential localization of leukocyte subsets is mediated in part by their differential expression of chemokine receptors (4). For example, activated/memory T lymphocytes display a higher density of the CC chemokine receptor CCR5 than do resting/naive T lymphocytes and, as a result, show greater chemotaxis in response to CC chemokines such as macrophage inflammatory protein-1 (MIP-1) (5). Conversely, naive T lymphocytes express greater levels of the CXC chemokine receptor CXCR4 than do memory T lymphocytes and, as a result, show higher chemotaxis in response to CXC chemokines such as SDF-1 (5). Thus, differential chemokine receptor expression may play a critical role in the differential trafficking patterns of naive versus mature T lymphocytes. Similar dichotomies in chemokine receptor expression appear to distinguish CD4+ T lymphocytes supporting cellular immune responses from those that foster humoral responses (6–8).

Chemokine receptor expression has received a great deal of attention following the discovery that certain chemokine receptors function in conjunction with CD4 to mediate HIV infection of human cells (9). CXCR4 was initially identified as a coreceptor for T cell-tropic strains of HIV-1 (10), and CCR5 and several other CC chemokine receptors were subsequently identified as coreceptors for macrophage-tropic strains of HIV-1 (11–13). CXCR4 can also mediate CD4-independent infection of human cells by HIV-2 (14, 15). Varying levels of chemokine receptor expression can influence cellular susceptibility to HIV infection (16, 17), and chemokines and anti-receptor Abs can block HIV infection under certain circumstances (18–23).

Because chemokine receptors play a critical role in both normal immune system function and HIV pathogenesis, understanding the factors that regulate their expression may provide options for therapeutic intervention in a variety of pathophysiologic settings (1–4). In addition to differential expression across distinct leukocyte subpopulations, chemokine receptor density is also altered in the presence of its natural ligand (24), over the course of mitosis (5, 24, 25), and during cellular maturation (26). Cytokines can alter the expression of some CC chemokine receptors (27, 28) although their effects on lymphocyte CXC chemokine receptors remain poorly defined. Chemokine receptors are members of a diverse family of cell surface receptors characterized by a seven-transmembrane serpentine structure and signal transduction via heterotrimeric guanine nucleotide binding proteins (4, 29, 30). Cell surface expression of G-protein-linked receptors is regulated both by gene expression and by continual recirculation of receptors between the cell surface and endosomal compartments (24, 31). Distinct signaling pathways govern these two modes of receptor regulation. For example, whereas certain mitogenic stimuli suppress CXCR4 expression by increasing receptor internalization via the protein kinase C (PKC) signaling cascade, this pathway does not appear to mediate ligand-induced suppression of CXCR4 (24).

With the exception of mitogenic stimuli and ligand-induced down-regulation, little is known about the role of extracellular factors in regulating lymphocyte expression of CXC chemokine receptors. However, the pronounced effects of cytokines, growth factors, and hormones on lymphocyte localization (3, 32–34) suggest
that extracellular factors may well play an important role in chemokine receptor expression. One major mode of extracellular influence on lymphocyte function comes from a class of secreted molecules that activate the cellular cAMP-dependent protein kinase A (PKA) (35). The cAMP-PKA signaling pathway represents a common second messenger system for a variety of distinct receptors that bind a diverse array of hormones, neurotransmitters, and peptide-signaling molecules (e.g., E series PGs, histamine, catecholamines, neurohypophyseal hormones (such as corticotropin-releasing factor and vasopressin), and pro-opiomelanocortin-derived peptides (such as adrenocorticotropic hormone)) (30). As a result of cAMP signaling, PKA phosphorylates multiple intracellular substrates, including elements of other signaling pathways (e.g., phospholipase Cβ1) and regulators of gene transcription (e.g., cAMP response element binding protein (CREB)). The cAMP-PKA signaling pathway plays a critical “switching” role in a variety of physiologic settings ranging from glycolysis to ontogenic differentiation. In the immune system, cAMP signaling modulates cellular activation (35, 36) and alters cytokine production profiles (37–41). cAMP-inducing stimuli can also alter leukocyte traffic and localization (33, 34, 42), although the molecular basis for such effects is not fully understood.

In the present study, we explore the role of the cAMP-PKA signaling pathway in modulating lymphocyte expression of CXCR4, the receptor for the chemokine SDF-1 (19, 23). In contrast to most other chemokine systems, which recruit cells to sites of inflammation, the SDF-1/CXCR4 system is believed to localize cells to lymphoid organs (43–45). Consistent with this function, CXCR4 is richly expressed on hemopoietic progenitor cells and immature thymocytes, on fully differentiated but antigenically naive B and T lymphocytes, and on monocytes and cultured dendritic cells (5, 26, 46). Lymphocyte CXCR4 expression is down-regulated during cellular activation (24), and CXCR4 is virtually absent from NK cells, neutrophils, eosinophils, and freshly isolated Langherans cells (5, 21, 47). Here, we examine the role of cAMP in regulating CXCR4 expression on both activated and unstimulated PBMC. CD4+ cells represent a special focus of attention due to their potential vulnerability to HIV infection. In addition, we consider the functional implications of cAMP regulation of CXCR4 for ligand-induced internalization, SDF-1-induced chemotaxis, and vulnerability to infection with CXCR4-tropic HIV-1.

Materials and Methods

Cell culture and activation

Healthy donor PBMC were isolated by Ficoll density gradient and cultured at 3 x 10^6/ml in RPMI 1640 supplemented with 10% (v) human AB serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine, at 37°C in an atmosphere of 5% CO2. PKA-activating ligands were added once, at the beginning of culture, and included the membrane-permeable cAMP analogue, N6-2′-O-dibutyl adenosine-3′,5′-cyclic monophosphate (dbcAMP), the adenylyl cyclase activator Forskolin, and the physiologic cAMP inducers PGE2, histamine (H), adrenocorticotropic hormone (ACTH), epinephrine (E), and norepinephrine (NE) (all from Sigma, St. Louis, MO). Activated cells were costimulated with Abs to CD3 (0.1 µg/ml of suspension solution was aspirated, and 10 µl of anti-CD4 Ab according to the manufacturer’s protocol, fixed by suspension in 1 ml 4% paraformaldehyde, pelleted, and resuspended in 1 ml of saponin buffer (0.1% w/v saponin plus 0.05% azide in HBSS) for permeabilization. Unpermeabilized cells were suspended in 1 ml HBSS. All cells were then pelleted, 800 µl of suspension solution was aspirated, and 10 µl of anti-CXCR4 Ab was added. Following 30 min of incubation at room temperature, cells were washed once in 1.5 ml of either saponin buffer (permeabilized cells) or HBSS (unpermeabilized cells), pelleted, and resuspended in PBS for flow cytometry. Fluorescence intensity was measured by FACScan analysis gated to exclude dead cells and debris on the basis of forward- vs side-scatter profiles. Intracellular CXCR4 compartmentalization was quantified by subtracting the mean fluorescence intensity of anti-CXCR4 Ab binding on unpermeabilized cells (extracellular CXCR4) from that of permeabilized cells (intracellular plus extracellular CXCR4). Statistical significance of differences in intracellular compartmentalization was assessed by t test. Non-specific Ab binding was assessed by parallel staining with an isotype-matched phycoerythrin-conjugated anti-keyhole limpet hemocyanin (KLH) Ab and did not differ across cAMP-treated and -untreated cells. Analysis subtracting non-specific fluorescence intensity from anti-CXCR4 fluorescence intensity produced similar results.

Ligand-induced down-regulation

CXCR4 down-regulation in response to exogenous ligand was assessed by flow cytometric quantitation of cell surface CXCR4 expression on co-stimulated PBMC (as described above) at 5 and 30 min after addition of...
100 ng/ml SDF-1α (R&D Systems). Effects of ligand-induced internalization were quantified by expressing CXCR4 mean fluorescence intensity on SDF-1α-treated cells as a fraction of CXCR4 mean fluorescence intensity on untreated cells.

Recirculation of CXCR4 to the cell surface following SDF-1α-induced internalization was assessed by flow cytometric quantification of CXCR4 expression on costimulated PBMC at 0, 20 and 60 min after washing SDF-1α-treated cells twice in PBS and resuspending them in fresh culture medium. Surface expression recovery rates were estimated by linear regression of CXCR4 mean fluorescence intensity vs time since SDF-1α washout, and recovery rates on costimulated cells and cells costimulated in the presence of dbcAMP were compared by t test.

**Chemotaxis**

Chemotactic response to SDF-1α was measured by a membrane transmigration assay in which 5 × 10⁵ PBMC in 100 μl medium (RPMI + 0.25% BSA) were loaded into the upper chamber of a Transwell insert with a 5-μm pore size polycarbonate membrane separating cells from a lower chamber containing graded concentrations of SDF-1α. The number of cells transmigrating during 2 h of incubation was quantified by FACSc (gating out dead cells and debris on the basis of forward- and side-scatter profiles), and SDF-1α-induced chemotaxis was calculated by subtracting background transmigration (0 ng/ml SDF-1α) and standardizing relative to a 500-μl sample containing 10% of the input cell number. SDF-1α-induced chemokinesis (ambidirectional motility) was assessed by transmigration rates in the presence of 300 ng/ml SDF-1α in both upper and lower chambers (45). Statistical significance of differential chemotaxis rates was assessed by t test and comparison of dose-response curves by linear regression.

**HIV-1 infection and expression**

Healthy donor PBMC were infected with CXCR4-tropic HIV-1NL-Δ3 (49) (0.05 infectious units per cell) for 30 min in the presence of 10 μg/ml polybrene. Following infection, cells were washed twice and costimulated as described above. To prevent viral spread, postinfection medium was changed for 25 cycles in parallel with control standards consisting of linearized HIV provirus and internalized HIV-1 DNA and known quantities of cellular DNA. Radiolabeled amplified products were resolved on a 6% polyacrylamide gel and quantified by radioanalytic image analysis in comparison with standard curves. To ensure that viral spread did not occur, proviral DNA was also quantified at 48 h postinfection. In no case did the fraction of provirus-bearing cells at 48 h exceed that at 14 h postinfection.

HIV-1 gene expression was quantified by flow cytometric detection of a reporter gene product from a genetically altered HIV-1NL-Δ3-ΔHIV-1NL-HSAS was created by cloning the murine heat-stable Ag (HSA) gene into a vacancy created by deleting nucleotides 5625 through 5742 in the vpr gene of HIV-1NL-Δ3 (51). Upon transcription and translation of HIV-1NL-HSAS, murine HSA is expressed on the cell surface and can be quantified by flow cytometric assessment of anti-murine CD24 MAb binding (FITC-conjugated M1/69; Pharmingen). This virus is pathogenic in vivo in SCID mice, and HSA reporter gene expression correlates closely with other means of verifying HIV infection, including PCR assessment of proviral DNA and flow cytometric quantitation of intracellular p24 (51).

**Results**

**cAMP up-regulates CXCR4 expression on both resting and activated lymphocytes**

Activation of the lymphocyte cAMP-PKA signaling pathway alters lymphocyte traffic and localization (33, 34, 42) and increases HIV replication (52–54), although the mechanisms underlying such effects remain incompletely understood. To determine whether altered expression of chemokine receptors might play a role, we examined the effect of cAMP in regulating cell surface expression of CXCR4. On unstimulated PBMC, CXCR4 expression spontaneously increased over the first 12 h of culture and subsequently remained stable for up to 3 days (Fig. 1). Equivalent culture in the presence of 100–300 μM dbcAMP significantly increased CXCR4 cell surface expression by 12 h, with CXCR4 levels declining gradually thereafter but remaining significantly elevated above those of untreated cells (Fig. 1). dbcAMP treatment did not significantly affect CXCR4 expression levels during the first 6 h of culture as receptor expression spontaneously increased on both untreated and dbcAMP-treated cells (data not shown). Pronounced effects of dbcAMP on CXCR4 expression were observed only once CXCR4 levels reached a steady state (between 6 and 12 h of culture), suggesting that cAMP regulates the constitutive receptor set point rather than the receptor generation process itself. cAMP up-regulation of CXCR4 was dose dependent (Fig. 1, Table 1), and cAMP-inducing ligands produced similar effects, with 100 μM Forskolin increasing CXCR4 cell surface expression by up to sixfold at 24 h (Table 1). CXCR4 expression was also increased by a variety of physiologic cAMP-inducing ligands, including PGE₂, histamine, ACTH, and the catecholamines epinephrine and NE (Table 1). Similar dynamics were observed in CD4⁺, CD8⁺, and CD19⁺ subsets (Fig. 1) although with differing kinetics in each subset. On CD4⁺ cells, cAMP-induced CXCR4 expression peaked at 12 h and declined gradually thereafter, whereas peak expression on CD8⁺ and CD19⁺ cells occurred at 24 h, followed by a rapid decline toward baseline levels (Fig. 1). At all time points observed, CD19⁺ cells showed significantly greater levels of CXCR4 expression than did CD4⁺ or CD8⁺ cells (all p < 0.0001). In contrast to cAMP effects on lymphocyte CXCR4, cAMP significantly suppressed CXCR4 expression on CD14⁺ cells (Fig. 1). cAMP did not significantly alter cell surface CD4 expression at any time point examined (data not shown).

Following CD3/CD28 costimulation, PBMC cell surface expression of CXCR4 was suppressed by approximately 70% relative to unstimulated PBMC (Fig. 1) (p < 0.001 at each time point). Equivalent costimulation in the presence of dbcAMP significantly up-regulated CXCR4, with peak expression levels significantly exceeding those of both costimulated PBMC and unstimulated PBMC in the absence of cAMP (p < 0.0001) (Fig. 1). Elevated CXCR4 cell surface expression was observed by 12 h of costimulation, peaked at 24 h, and declined gradually back toward unstimulated levels at 48 and 72 h (Fig. 1). As with unstimulated cells, cAMP-inducing ligands also up-regulated CXCR4 expression on costimulated PBMC (Table 1), with 100 μM Forskolin increasing CXCR4 levels by 10-fold. Similar CXCR4 expression kinetics were observed for CD4⁺, CD8⁺, and CD19⁺ cells, with CXCR4 levels on CD19⁺ cells significantly exceeding those on CD4⁺ and CD8⁺ cells (Fig. 1). In the absence of cAMP, CXCR4 expression on CD19⁺ cells from costimulated cultures was significantly suppressed relative to CD19⁺ cells from unstimulated cultures, implying some degree of cross regulation by T cell activation (since CD19⁺ cells should not be directly activated by CD3/CD28 costimulation). As with unstimulated cells, cAMP significantly suppressed CXCR4 expression on CD14⁺ cells (Fig. 1) and did not significantly alter CD4 expression at any time point examined (data not shown).

**cAMP does not alter CXCR4 gene expression**

To determine whether cAMP-induced up-regulation of cell surface CXCR4 was mediated by increased gene expression, receptor mRNA levels were quantified by RT-PCR following 20 h of culture (preceding the period of peak difference in receptor expression). dbcAMP failed to significantly increase CXCR4 mRNA levels in either resting or costimulated PBMC (Fig. 2). Across five experiments, the average CXCR4 mRNA level in cAMP-treated
cells differed from that of untreated cells by less than 5%. Moreover, dbcAMP-induced up-regulation of CXCR4 was not abrogated by the protein synthesis inhibitor cyclohexamide (data not shown), confirming that cAMP up-regulation of CXCR4 cell surface expression is not mediated by increased CXCR4 gene expression.

**cAMP reduces CXCR4 internalization**

To determine whether altered receptor trafficking might play a role in cAMP up-regulation of CXCR4 cell surface expression, receptor internalization rates were quantified by flow cytometric measurement of internalized anti-CXCR4 Ab following removal of surface-bound Ab by acid washing. Residual Ab binding reflects internalized receptors not exposed to extracellular low pH (24). Low pH washing efficiently removed cell surface-bound Ab, as demonstrated by the abrogation of anti-CD4 Ab binding (Fig. 3A) and the abrogation of anti-CXCR4 Ab binding in cells incubated at 4°C to prevent internalization (Fig. 3B). Under normal incubation conditions (37°C), acid-resistant anti-CXCR4 Ab binding averaged 20% of total CXCR4 fluorescence intensity at 60 min (Fig. 3, C and D). Parallel incubation in the presence of dbcAMP decreased acid-resistant anti-CXCR4 Ab binding by 39%, to an average of 12% of total fluorescence intensity at 60 min (p < 0.001). In contrast, dbcAMP did not significantly alter acid-resistant anti-CD4 binding (Fig. 3E), indicating a specific effect of cAMP on CXCR4 internalization. Similar effects were observed in isolated CD4+ T cell cultures, with dbcAMP decreasing acid stable anti-CXCR4 binding by an average of 44% (data not shown). This cAMP appears to up-regulate cell surface expression of CXCR4 in part by reducing receptor internalization rates.

Consistent with reduced internalization, flow cytometric analysis of CXCR4 compartimentalization indicated that cAMP decreased the fraction of CXCR4 in intracellular compartments. Intracellular CXCR4 constituted an average of 38% of total CXCR4 fluorescence intensity on costimulated PBMC, with 100 µM dbcAMP reducing this value by more than 50%, to an average of 18% across four experiments (p = 0.004). DbcAMP did not significantly affect total CXCR4 fluorescence intensity (intracellular plus extracellular; mean = 736 ± 330 vs 822 ± 216 fluorescence units for costimulated PBMC vs PBMC costimulated in the presence of 100 µM dbcAMP; p = ns), suggesting that cAMP alters CXCR4 cell surface expression primarily by altering receptor compartmentalization rather than by increasing the size of the total CXCR4 receptor pool. Consistent with this mechanism, cAMP-induced down-regulation of CXCR4 on CD14+ cells was accompanied by a significant increase in the fraction of CXCR4 in intracellular compartments (mean = 31% in costimulated cultures vs 80% in cultures costimulated in the presence of dbcAMP, p = 0.049) without any change in total CXCR4 fluorescence intensity.

### Table 1. Effects of cAMP and cAMP-inducing agents on CXCR4 expression on PBMC

<table>
<thead>
<tr>
<th>Agent</th>
<th>Unstimulated</th>
<th>CD3/CD28 Costimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>db-cAMP dose-response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>191% (1%)</td>
<td>487% (3%)</td>
</tr>
<tr>
<td>200 µM</td>
<td>302% (1%)</td>
<td>729% (13%)</td>
</tr>
<tr>
<td>300 µM</td>
<td>347% (1%)</td>
<td>826% (1%)</td>
</tr>
<tr>
<td>cAMP-inducing agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forskolin (10^-5 M)</td>
<td>607% (3%)</td>
<td>1001% (10%)</td>
</tr>
<tr>
<td>Prostaglandin E2 (10^-4 M)</td>
<td>198% (2%)</td>
<td>235% (2%)</td>
</tr>
<tr>
<td>Norepinephrine (10^-3 M)</td>
<td>151% (1%)</td>
<td>196% (5%)</td>
</tr>
<tr>
<td>Epinephrine (10^-3 M)</td>
<td>145% (1%)</td>
<td>159% (3%)</td>
</tr>
<tr>
<td>Histamine (10^-5 M)</td>
<td>184% (2%)</td>
<td>152% (4%)</td>
</tr>
</tbody>
</table>

a Data represent mean (SE) percentage increase in CXCR4 fluorescence intensity relative to no-cAMP controls following 24 h of culture (three experiments). All values differ significantly from control value of 100% (p < .0001).

b ND, not done.
FIGURE 2. Effect of cAMP on CXCR4 mRNA expression. RT-PCR was used to quantify CXCR4 mRNA expression in unstimulated and CD3/CD28 costimulated PBMC cultured for 20 h in the presence of 0 or 100 μM dbcAMP. Parallel determination of β-actin mRNA verified equivalent mRNA loading. Samples were quantified by densitometric image analysis in comparison with dilution-titrated PBMC mRNA standards. Across five experiments, CXCR4 mRNA levels in dbcAMP-treated cultures differed by less than 5% from those of untreated cultures.

cAMP-induced CXCR4 up-regulation counteracts ligand-induced down-regulation

CXCR4 undergoes rapid internalization after binding its natural ligand, SDF-1 (18, 31). To determine whether cAMP-induced up-regulation of CXCR4 cell surface expression can offset ligand-induced down-regulation, 100 ng/ml SDF-1α was added to PBMC costimulated for 24 h in the presence or absence of dbcAMP. As shown in Table II, 100 ng/ml SDF-1α suppressed CXCR4 cell surface expression within 5 min, and this suppression persisted for at least 30 min (p = 0.007). dbcAMP (10 μM) (Table II) offset the effects of SDF-1α and restored cell surface CXCR4 expression to levels that did not differ significantly from those observed on cells costimulated in the absence of SDF-1α (p = 0.277). dbcAMP (100 μM) (Table II) counteracted ligand-induced internalization effects and elevated CXCR4 expression to levels significantly greater than those observed on costimulated PBMC (untreated with either SDF-1α or dbcAMP; p = 0.020). Thus cAMP up-regulation of CXCR4 counteracts ligand-induced CXCR4 suppression.

FIGURE 3. Effect of cAMP on CXCR4 internalization. CXCR4 internalization was quantified by flow cytometric assessment of anti-CXCR4 Ab binding following acid stripping of cell surface-bound Ab. To assure that acid washing effectively stripped surface-bound Abs, anti-CD4 Ab binding was measured following wash at pH 7 (A, top) or pH 3 (A, bottom) (CD4 internalization is minimal on normal PBMC). To confirm the effect of acid washing on anti-CXCR4 surface binding, cells were maintained at 4°C to inhibit receptor internalization for 60 min before wash at pH 7 (B, top) or pH 3 (B, bottom). To quantify CXCR4 internalization during 37°C incubation, acid-resistant Ab binding (internalized) was expressed as a fraction of total Ab binding (internal plus external) (C) over 1–60 min of incubation. dbcAMP (100 μM) suppressed CXCR4 60-min internalization rates by an average of 39% across four experiments (p = 0.001) (D) (SE falls within the plotting symbol for data points lacking error bars). In contrast, CD4 internalization rates were not significantly affected (E). Data represent the mean (SE) across four experiments, with statistical significance of comparisons at specific time points indicated.

Ligand-induced CXCR4 suppression is rapidly reversed upon removal of SDF-1α, suggesting that such effects are mediated by internalization into endosomal compartments and subsequent recirculation to the cell surface (31). To determine whether cAMP influences CXCR4 reexternalization rates, CXCR4 cell surface expression was monitored on cells treated with 100 ng/ml SDF-1α for 1 h and then washed twice and resuspended in fresh medium. Following suppression to 57% of pre-SDF-1α levels, CXCR4 expression levels on costimulated PBMC recovered by an average of 0.34% per minute during the first 60 min following SDF-1α removal. In cells costimulated in the presence of dbcAMP, CXCR4 recovery rates increased to 0.67% per minute (p = 0.029). Thus cAMP up-regulation of cell surface CXCR4 can influence both ligand-induced receptor internalization and reexternalization following ligand removal.

cAMP-induced up-regulation of CXCR4 increases SDF-1α-induced chemotaxis

To explore the functional consequences of cAMP-induced up-regulation of CXCR4, lymphocyte chemotactic response to SDF-1α was assessed in membrane transmigration assays. In PBMC costimulated for 24 h, SDF-1α produced dose-dependent increases in lymphocyte migration, with the fraction of transmigrating cells increasing by 5.8% with each 100 ng/ml increase in SDF-1α concentration (Fig. 4). Costimulation in the presence of 100 μM dbcAMP increased transmigration rates by approximately threefold (to 15.8% per 100 ng/ml, p < 0.0001) (Fig. 4). More pronounced effects were observed using the adenyl cyclase activator Forskolin (100 μM), which increased transmigration rates by fivefold (28.6% per 100 ng/ml, p < 0.0001; data not shown). Physiologic cAMP-inducing ligands also increased SDF-1α-induced chemotaxis, with 10 μM NE or PGE2 each approximately doubling transmigration rates (p = 0.004).

To distinguish cAMP’s effects on migration along a chemokine gradient (chemotaxis) from chemokine-induced alterations in random motility (chemokinesis), transmigration rates were assessed in the presence of 300 ng/ml SDF-1α on both sides of the membrane. PBMC showed minimal chemokinetic response to SDF-1α (transmigration rates differed from those of no-SDF controls by less than 5%), and dbcAMP did not significantly alter these effects (data not
Table II. Effect of cAMP on SDF-1-induced CXCR4 down-regulation

<table>
<thead>
<tr>
<th>SDF-1 (ng/ml)</th>
<th>db-cAMP (μM)</th>
<th>Time</th>
<th>5 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td></td>
<td>145 (100%)</td>
<td>156 (100%)</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td></td>
<td>104 (71%)</td>
<td>88 (56%)</td>
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<tr>
<td>100</td>
<td>10</td>
<td></td>
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<td>134 (86%)</td>
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<tr>
<td>100</td>
<td>100</td>
<td></td>
<td>445 (307%)</td>
<td>314 (201%)</td>
</tr>
</tbody>
</table>

* Data represent mean fluorescence intensity of anti-CXCR4 Ab binding to CD3/CD28-costimulated PBMC at indicated times following resuspension of cells in fresh medium containing indicated concentrations of SDF-1α. Parenthesized values represent CXCR4 fluorescence relative to costimulated cells incubated in the absence of SDF-1α.

shown). Thus cAMP up-regulation of SDF-1α-induced chemotaxis was not mediated by increased random mobility.

cAMP-induced up-regulation of CXCR4 increases vulnerability of CD4+ cells to HIV-1 infection

PCR was used to quantify reverse-transcribed proviral DNA at 14 h following infection by CXCR4-tropic HIV-1NL4-3. When PBMC were infected following 24 h of costimulation, an average of 4% bore proviral DNA 14 h later (Fig. 5A). Costimulation in the presence of dbcAMP increased proviral penetrance by approximately eightfold (Fig. 5A). These cAMP-induced increases in HIV-1 infectivity were mediated specifically by altered CXCR4 expression, as demonstrated by the fact that cAMP effects on proviral penetrance could be blocked by preincubating cells in the presence of the CXCR4-specific ligand SDF-1α (1 mM), but not by parallel preincubation in the presence of the CCR5 ligand MIP-1α (1 mMF; see Fig. 5A). Thus cAMP-induced up-regulation of CXCR4 expression increases cellular vulnerability to infection by CXCR4-tropic HIV-1.

Studies using the HIV-1NL4-HSA reporter virus (murine HSA gene cloned into a vpr-deleted HIV-1NL4-3; Ref 51) showed that cAMP-induced increases in proviral penetrance were accompanied by an increase in the fraction of cells expressing viral genes at 48 h postinfection (Fig. 5B). Similar effects were observed using physiologic inducers of cAMP (e.g., 10 μM NE increased HIV-1 gene expression to 230% of costimulated control levels, p = 0.022). To ensure that cAMP-induced increases in HIV-1 gene expression did not reflect enhanced viral spread, cells were cultured in the presence of an HIV-1 protease inhibitor (100 nM Indinavir). PCR analysis of proviral load at 48 h postinfection confirmed that antiretroviral treatment prevented spread of provirus beyond cells infected by 14 h (data not shown). Thus cAMP-induced up-regulation of cell surface CXCR4 expression renders T lymphocytes more vulnerable to productive infection by CXCR4-tropic HIV-1.

Kinetic analysis of HSA expression as a function of CXCR4 density indicated that HSA initially appeared on cells expressing high levels of CXCR4 (mean fluorescence intensity > 1000; data not shown). As HSA intensity increased over time, CXCR4 levels declined, with average expression dropping below 100 fluorescence intensity units on cells showing maximal HSA expression (Fig. 5B). Such results may arise from several possible mechanisms, including down-regulation of CXCR4 expression by HIV gene expression and differential survival of cells expressing high vs low levels of CXCR4.

Discussion

Activation of the cAMP-PKA signaling pathway alters lymphocyte traffic and localization (33, 34, 42), but the mechanisms underlying such effects remain poorly understood. Here, we show that cAMP-PKA signaling can increase cell surface expression of the CXC chemokine receptor CXCR4 by 6- to 10-fold on unstimulated and CD3/CD28-costimulated lymphocytes. These effects are mediated primarily by cAMP-induced alterations in the balance of CXCR4 expressed in intracellular vs extracellular compartments. We also show that cAMP up-regulation of CXCR4 can offset the effects of ligand-induced internalization and accelerate recovery of cell surface CXCR4 expression following release from ligand-induced suppression. Functional results of cAMP-induced up-regulation of CXCR4 include increased chemotaxis in response to SDF-1α and increased vulnerability to infection by CXCR4-tropic HIV-1. cAMP up-regulates CXCR4 expression on both CD4+ and CD8+ T lymphocytes as well as on CD19+ B cells. In contrast to these effects on lymphocytes, cAMP suppresses CXCR4 expression on CD14+ cells by promoting CXCR4 internalization. These data imply that the diverse family of extracellular factors that activate the cAMP-PKA signaling pathway may function as natural modulators of leukocyte traffic and localization and incidentally render CD4+ T cells more vulnerable to infection with CXCR4-tropic HIV-1.

Because SDF-1 also regulates aspects of lymphopoiesis and tissue development (55, 56), cAMP regulation of CXCR4 expression may have additional implications for cellular maturation and morphogenesis.

The present results suggest that cAMP may modulate lymphocyte circulation patterns by altering expression of chemokine receptors. Such results are consistent with evidence that cAMP-inducing ligands can selectively mobilize specific lymphocyte subsets into circulation while retaining other subsets in lymphoid organs (33, 34, 42). cAMP-inducing ligands are distributed both systematically via the endocrine system and locally via paracrine secretion from cells of the nervous and immune systems. Potential paracrine activators of the lymphocyte cAMP pathway in vivo include PGs and histamine secreted by myeloid cells at sites of inflammation (57, 58), catecholamines secreted by sympathetic nervous system neurons terminating in lymphoid organ parenchymal tissues (59), and ACTH secreted by activated lymphocytes (60). Signaling via the cAMP-PKA pathway may interact with other CXCR4-modulators to modify cellular localization in response to...
antigenic signals. For example, CXCR4 expression on T lymphocytes typically declines following cellular activation (Refs. 24 and 31, and Fig. 1), and this effect is particularly pronounced for CD45RA+ (naive) cells. This regulatory pathway may facilitate migration of activated cells out of lymphoid organs and into peripheral tissue sites (5). However, cAMP-inducing ligands may counteract this effect and thus promote retention of activated lymphocytes in areas of high SDF-1 concentration.

CXCR4 plays an important role in HIV pathogenesis by functioning in conjunction with CD4 as a coreceptor for virulent syncytium-inducing viral strains (9). Activation of the lymphocyte cAMP-PKA signaling pathway can accelerate HIV-1 replication (52–54). The present data suggest that cAMP effects on HIV replication may be mediated in part by increased cellular vulnerability to HIV infection as a function of up-regulated CXCR4 expression. Such effects may have significant clinical implications since the emergence of CXCR4-tropic HIV strains is associated with progression from chronic infection to the development of life-threatening illness (61, 62). Several cAMP-inducing mediators are increased during HIV infection (e.g., PGE; Ref. 63), and exogenous HIV-1 proteins can increase intracellular cAMP levels (64, 65). Such effects could conceivably promote disease progression by increasing T lymphocyte vulnerability to infection and thereby facilitating replication of CXCR4-tropic viral strains. Such a dynamic may undermine the use of cAMP-inducing agents to suppress chemokine receptors on myeloid cells (as demonstrated above) as an antiviral strategy (66).

The present data indicate that cAMP up-regulates lymphocyte CXCR4 expression primarily by altering receptor compartmentalization. The negligible effects of cAMP on total CXCR4 pool size...
and receptor gene expression are consistent with the absence of any known cAMP response element in the CXCR4 promoter (67, 68). Although cAMP up-regulation of cell surface CXCR4 is mediated by altered receptor trafficking, the molecular mechanism of these effects remains to be clarified. Activated PKA may directly phosphorylate the receptor system itself (as in other receptor families; Ref. 30), or it may interact with other signaling pathways that influence CXCR4 compartmentalization (69). Another outstanding question regards the teleologic rationale for receptor regulation by cAMP-inducing ligands. Many cAMP-inducing factors exert significant effects over lymphocyte function (e.g., PGs, catecholamines, ACTH), but their physiologic roles remain poorly understood. A more comprehensive understanding of both the sources and function of CXCR4 ligands may be helpful in elucidating the role of cAMP in the immune response more generally (1).

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References


