The CXC Chemokine Stromal Cell-Derived Factor Activates a G<sub>i</sub>-Coupled Phosphoinositide 3-Kinase in T Lymphocytes

Yannis Sotsios, Gillian C. Whittaker, John Westwick and Stephen G. Ward

*J Immunol* 1999; 163:5954-5963; ;
http://www.jimmunol.org/content/163/11/5954

References

This article cites 80 articles, 49 of which you can access for free at:
http://www.jimmunol.org/content/163/11/5954.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The CXC Chemokine Stromal Cell-Derived Factor Activates a G\textsubscript{i} -Coupled Phosphoinositide 3-Kinase in T Lymphocytes\textsuperscript{1}

Yannis Sotsios, Gillian C. Whittaker, John Westwick,\textsuperscript{2} and Stephen G. Ward\textsuperscript{3}

The cellular effects of stromal cell-derived factor-1 (SDF-1) are mediated primarily by binding to the CXC chemokine receptor-4. We report in this study that SDF-1 and its peptide analogues induce a concentration- and time-dependent accumulation of phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)\textsubscript{3}) in Jurkat cells. This SDF-1-stimulated generation of D-3 phosphoinositide lipids was inhibited by pretreatment of the cells with an SDF-1 peptide antagonist or an anti-CXCR4 Ab. In addition, the phosphoinositide 3 (PI 3)-kinase inhibitors wortmannin and LY294002, as well as the G\textsubscript{i} protein inhibitor pertussis toxin, also inhibited the SDF-1-stimulated accumulation of PtdIns(3,4,5)\textsubscript{3}. The effects of SDF-1 on D-3 phosphoinositide lipid accumulation correlated well with activation of the known PI 3-kinase effector protein kinase B, which was also inhibited by wortmannin and pertussis toxin. Concentrations of PI 3-kinase inhibitors, sufficient to inhibit PtdIns(3,4,5)\textsubscript{3} accumulation, also inhibited chemotaxis of Jurkat and peripheral blood-derived T lymphocytes in response to SDF-1. In contrast, SDF-1-stimulated actin polymerization was only partially inhibited by PI 3-kinase inhibitors, suggesting that while chemotaxis is fully dependent on PI 3-kinase activation, actin polymerization requires additional biochemical inputs. Finally, SDF-1-stimulated extracellular signal-related kinase (ERK)-1/2 mitogen-activated protein kinase activation was inhibited by PI 3-kinase inhibitors, suggesting that while chemotaxis is fully dependent on PI 3-kinase activation, ERK1/2 activation is dependent on PI 3-kinase activation, and both biochemical events are involved in the regulation of SDF-1-stimulated chemotaxis. The Journal of Immunology, 1999, 163: 5954 –5963.

Chemokines are a rapidly growing superfamily of 8- to 10-kDa peptides that selectively attract and activate leukocyte populations (1–3). Recent interest in chemokines and their receptors has increased substantially as a result of their emerging role in immune and inflammatory responses, hemopoiesis, and HIV infection (1–5). Four classes of chemokines have been defined based on the arrangement of the conserved cysteine (C) residues of the mature proteins: the CXC or α-chemokines, CC or β-chemokines, C or γ-chemokines, and the CX\textsubscript{3}C or δ-chemokines (1, 3, 6).

Stromal cell-derived factor (SDF-1)\textsuperscript{4} was first described as a factor that is produced by bone marrow stromal cells and shown to induce proliferation of B cell progenitors and regulate B cell maturation (7). Two isoforms, SDF-1α and SDF-1β, have been identified that are encoded by a single gene and arise from alternative splicing (8). SDF-1α is widely expressed and is a highly efficacious chemoattractant for monocytes, T lymphocytes, and CD3\textsuperscript{+} human progenitor cells (7–11). SDF-1α is the biological ligand for the chemokine receptor CXCR4, a seven-transmembrane G protein-coupled receptor (12–16). CXCR4 is expressed on PBL, monocytes, thymocytes, pre-B cells, as well as dendritic and endothelial cells (17–21). Moreover, CXCR4 is the coreceptor for the binding of T-tropic HIV strains (5, 12, 14, 15). Accordingly, SDF-1α and its various analogues inhibit CXCR4-mediated HIV-1 infection in vitro (15, 22, 23). Consistent with the effects of SDF-1 on pre-B cell proliferation, knockout mice lacking SDF-1α show abnormalities in B cell lymphopoiesis, bone marrow myelopoiesis, and cerebellar neuron migration, and also have nonfatal ventricular septal defects (24). Similar defects have been reported in CXCR4\textsuperscript{−/−} mice, which also exhibit defective vascularization of the gastrointestinal tract (25–27).

While our understanding of the biological role of SDF-1 has increased substantially in recent years, relatively little is known about the signaling pathways that may mediate these effects. SDF-1 has been shown to elicit elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, in a number of settings (10, 15) and has also been reported to stimulate phosphorylation of both MEK-1 and ERK1/2 in several cell models (28–31). SDF-1 stimulation also enhanced tyrosine phosphorylation of focal adhesion complex components (including Pyk-2, paxillin, and Crk), increased NF-κB activity, and induced PI 3-kinase activity associated with antiphosphotyrosine immunoprecipitates (29, 31). Thus, SDF-1 can couple to distinct signaling pathways that may mediate cell growth, migration, and transcriptional activation.

The prototypical class 1\textsubscript{A} PI 3-kinase consists of an 85-kDa regulatory subunit (responsible for protein-protein interactions via Src homology 2 domain interaction with phosphotyrosine residues), and a catalytic 110-kDa subunit (32). A distinct lipid kinase termed PI 3-kinase-γ is activated by G protein-coupled receptors, and this is the only characterized member of the class 1\textsubscript{A} G protein-coupled PI 3-kinase family, consisting of a unique 101-kDa regulatory subunit and a distinct 110-kDa catalytic subunit termed...
p110γ (32–34). Nevertheless, there is some evidence that G protein-coupled receptors such as FMLP receptors are also able to activate the p85/p110 PI 3-kinase (35, 36). In this respect, the p85/p110 heterodimer has been demonstrated to be synergistically activated by the βy subunits of G proteins and by phosphotyrosyl peptides (36). The class I PI 3-kinases can potentially generate three lipid products, namely phosphatidylinositol-(3)-monophosphate (PtdIns(3)P), phosphatidylinositol-(3,4)-bisphosphate (PtdIns(3,4)P), and phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P), which are collectively known as D-3 phosphoinositide lipids (reviewed in Refs. 37 and 38). In addition, both the p85/p110 heterodimer and PI 3-kinase-y exhibit dual specificity as both a lipid kinase and a serine protein kinase (39, 40). At present, both PtdIns(3,4)P2 and PtdIns(3,4,5)P3 can be regarded as signaling molecules, whereas PtdIns(3)P is thought to regulate membrane trafficking (37, 38). PI 3-kinase(s) is now regarded as an important intracellular signal that is upstream of a variety of responses including insulin-stimulated glucose uptake (41), membrane ruffling (42), and superoxide production (43). Moreover, activation of a number of downstream signaling proteins is known to be regulated by PI 3-kinase and its lipid products including protein kinase B (PKB), p70S6 kinase, and Rac (44–46).

Given the functional role of SDF-1 in chemotaxis (7–11), it is interesting to note that the PI 3-kinase inhibitor wortmannin inhibits SDF-stimulated chemotaxis of CXCR4-expressing pre-B cells (31) as well as chemotaxis of several other cell types in response to other CXC chemokines (e.g., IL-8) (47) or CC chemokines (e.g., RANTES and monocyte-chemoattractant protein-1) (48, 49). In this study, therefore, we have investigated the possible involvement of PI 3-kinase(s) in SDF-1 signal transduction and chemotaxis in T lymphocytes.

Materials and Methods

Reagents

Human rSDF-1α was purchased from PeproTech (Rocky Hill, NJ). SDF-1 peptide analogues were a kind gift of Ian Clark-Lewis (University of British Columbia). The anti-CXCR4 mAb 12G5 (50) was obtained from the National Institute of Health AIDS Research and Reference Reagent Program. The goat anti-PI 3-kinase-y polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All cell culture reagents and per- tussis toxin were purchased from Life Technologies (Paisley, U.K.). Wortmannin and standard phosphatidylinositol lipids were purchased from Sigma (Poole, Dorset, U.K.). [32P]Orthophosphate (8500–9120 Ci/mol) was from DuPont-NEN (Boston, MA). All other reagents were purchased from Sigma.

Cell culture

The human leukemic T cell line Jurkat expressing CXCR4 was cultured in humidified incubators at 37°C, 5% (v/v) CO2 in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 µg/ml amphotericin B. CHO cells transfected with B7.1 cDNA (CHO-B7.1) were established and maintained previously described (51).

T cell purification and T lymphoblast preparation

Heparinized blood samples were separated on a Histopaque (1.078) density gradient. PBMC were removed from the gradient, and purified T cells were obtained by negative selection, as described (51). Alternatively, PBMCs (106 cells/ml) were stimulated with staphylococcal enterotoxin B (1 µg/ml) for 72 h. The cells were washed and growth maintained by supplementing every 2 days with 0.1 ml IL-2. After 10 days, cells were deprived of IL-2 for at least 2 days and allowed to accumulate in the G0/G1 stage of the cell cycle (52).

Flow cytometry

Peripheral blood-derived T lymphocytes, IL-2-maintained T lymphoblasts, or Jurkat cells (2 × 107) were stained with 10 µg/ml anti-CXCR4 Ab 12G5 or IgG isotype control (IgG2a) for 45 min at 4°C, washed, and incubated for a further 45 min at 4°C with 10 µg/ml anti-IgG FITC secondary Ab. Cells were washed and subsequently analyzed using a Becton Dickinson (San Jose, CA) FACS Vantage; excitation λ 488 nm, emission λ 530 nm.

D-3 phosphoinositide lipid labeling, extraction, and HPLC separation

A total of 1 × 106 cells were labeled with 1 µCi [32P]Orthophosphate (8500–9120 Ci/mmole; DuPont-NEN), as described (53). 132P-labeled Jurkat cells were aliquoted at 1 × 106 ml and stimulated as described in the figure legends, and the phospholipids were extracted with 700 µl chloroform: methanol: H2O (32.6%:65.3%:2.1% v/v/v, respectively) (53). The samples were decylated and analyzed by anion-exchange HPLC analysis using a Partisphere SAX column (Whatman, Maidstone, Kent, U.K.) (53). The eluate was fed into a Canberra Packard A-500 Flo-One on-line radiode-tector, and the results were analyzed by the Flo-One data program (Radiomatic, Cambridge, MA), with peaks being compared with retention times for standards prepared from [3H]-labeled phosphoinositide lipids (Amersham-Pharmacien Biotech, Amersham, Bucks, U.K.) and 32P-labeled D-3 phosphoinositides described elsewhere (54).

Cell lysis and in vitro PI 3-kinase assays

A total of 1 × 106 cells/ml were equilibrated for 10 min at 37°C and then stimulated in RPMI 1640 medium, as described in the figure legends. Reactions were terminated by pelleting cells in a microfuge for 10 s, followed by addition of 0.5 ml of the supernatant and addition of 0.5 ml of the [32P]Orthophosphate (1% [v/v] Nonidet P-40, 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM iodoacetamide, 10 mM NaF, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 10 µg/ml β-glycerophosphate, and 1 mM sodium orthovanado- nate). Lysates were rotated at 4°C for 15 min, followed by centrifugation at 14,000 rpm. The supernatants were preclarified, and immunoprecipitation was performed using anti-PI 3-kinase γ Ab (3 µg/ml). Immunoprecipit- ates were washed and subjected to in vitro lipid kinase assay using a lipid mixture of 100 µl of 0.1 mg/ml PtdIns and 0.1 mg/ml phosphatidylerine dispersed by sonication in 25 mM HEPES, pH 7.4, and 1 mM EDTA (49). The reaction was initiated by the addition of 10 µCi of [γ-32P]ATP (3000Ci/mmol; DuPont-NEN) and 100 µM ATP to the immunoprecipi- tates suspended in 80 µl of kinase buffer (5 mM MgCl2, 0.25 mM EDTA, 20 mM HEPES, pH 7.4). The reaction was terminated after 30 min and the phospholipids were then taken by TLC (49). The TLC plates were stained with iodine to confirm even extraction of substrate lipid between individual samples, and 32P-labeled PtdIns(3)P was visualized by autoradio- graphy (49).

Immunoblotting

Aliquots of cell lysate supernatant were boiled in Laemmli buffer and elec- trophoresed through 7.5% (v/v) acrylamide gels by SDS-PAGE, and the proteins were transferred onto nitrocellulose (Schleicher & Schuell, Keene, NH), as described previously (49). The blots were probed with a phosphospecific PKB Ab (0.5 µg/ml), which only has affin- ity for the active Ser473-phosphorylated forms of PKB (New England Biolabs, Woburn, MA) and proteins were visualized using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ) with a goat anti-rabbit Ig (0.1 µg/ml) conjugated with HRP as a secondary Ab. Where appropriate, blots were completely stripped of Abs by incubation at 55°C for 60 min with stripping solution (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10 mM 2-ME). After extensive washing, blots were reblocked, and total levels of PKB were detected by reprobing with 0.5 µg/ml anti-PKB Ab (New England Biolabs). Alternatively, cell lysates were separately probed with phosphoprotein-specific Abs detecting ERK1/2 (p44/p42) when phosphorylated at Thr202 and Tyr204, followed by stripping and reprobing with Abs to detect total levels of ERK1/2 (New England Biolabs).

Determination of [Ca2+]i

Jurkat cells and T lymphoblasts were suspended at 105 cells/ml in RPMI 1640 medium supplemented with 10% FCS and incubated for 30 min at 37°C with 2.5 µM fura-2 acetoxymethyl ester, as described previously (55). The fluorescence of a 2-ml cellular suspension was monitored with a Photon Technology International Delta Scan Fluorometer (dual excitation λ 340 and 380 nm, single emission λ 510 nm) at 37°C. Cytosolic free calcium concentration ([Ca2+]i) was determined by fluorescence using Photon Technology International software program (South Brunswick, NJ).

Chemotaxis assays

Chemotaxis was examined using a 96-well chemotaxis chamber (Neuro Probe, Cabin John, MD). The wells of the 96-well plate were filled with 380 µl of chemoattractant diluted in RPMI 1640 containing 0.1% BSA and...
covered with an adhesive polyvinylpyrrolidine-free polycarbonate mem-
brane (8 μM pore size). A total of 2 × 10^5 Jurkat cells or peripheral
blood-derived T lymphocytes were added to each upper well in a volume
of 200 μl, and the chamber was incubated at 37°C for 2 h. The cell sus-
pension was subsequently aspirated off, and 200 μl of Versene (Life Tech-
nologies) was added to each well. After 20-min incubation at 4°C, the
96-well plate and membrane were centrifuged at 1500 rpm for 10 min, the
supernatant was removed, and the cells were resuspended in 100 μl of
RPMI containing 0.1% BSA. Cell migration was assessed by adding 20 μl
of Cell Titer 96 AQ aqueous solution (Promega, Southampton, U.K.) to each
well. After a 2-h incubation at 37°C, the plate was read at λ490 nm,
subtracting the readings at a reference λ650 nm to reduce the background
contributed by nonspecific absorbance.

Actin polymerization
Purified T lymphocytes (2 × 10^6/0.5 ml) in RPMI 1640 were incubated at
37°C between 15 s and 30 min in the presence of SDF-1, and the cells were
then fixed by the addition of 0.5 ml of 7.4% formaldehyde in PBS. After
washing and permeabilization in 0.1% Triton solution in PBS for 10 min,
the cells were incubated with 100 μl of 0.3 μM FITC-phalloidin at 4°C for
30 min. The cells were then washed twice in PBS and resuspended in 500 μl of 1% paraformaldehyde/PBS solution. Data were analyzed on a Becton
Dickinson FACS Vantage, excitation λ488 nm, emission λ 530 nm.

Results
SDF-1 and its analogues stimulate the accumulation of
PtdIns(3,4,5)P_3 in Jurkat cells and T lymphoblasts
The leukemic T cell Jurkat has been used previously to investigate
biochemical responses to CXCR4 (28) and expresses high levels of
this receptor (Fig. 1A). Therefore, we have used ^32P-labeled Jurkat
cells with SDF-1 resulted in a significant concentration-dependent
accumulation of PtdIns(3,4,5)P_3 above resting levels (Fig. 2A). The maximum levels of PtdIns(3,4,5)P_3 accumulation following stimulation with SDF-1 were approximately one-half that observed in response to a maximal type stimulus for p85/p110 PI
3-kinase activation in Jurkat cells resulting from ligation of CD28 (54, 55). The SDF-1-induced increase in PtdIns(3,4,5)P_3 exhibited bell-shaped characteristics, with the maximum response observed
in the presence of 10 nM SDF-1 (Fig. 2A). Furthermore, the SDF-1-stimulated formation of PtdIns(3,4,5)P_3 was extremely rapid and
transient, because it was detectable 15 s after stimulation and had
returned toward basal levels 2–5 min after SDF-1 treatment (Fig. 2B).

A number of synthetic peptide analogues corresponding to the
N-terminal sequence of SDF-1 have been described that act as
either partial agonists or antagonist in chemotaxis and calcium
signaling assays (56). At concentrations previously demonstrated
to elicit a biochemical event such as elevation of [Ca^{2+}]_i (56), the
partial agonists SDF-1 1–9 and SDF-1 1–9 dimer are both effective
at eliciting modest accumulation of PtdIns(3,4,5)P_3, albeit at lower

![FIGURE 1. FACS analysis of CXCR4 receptor expression on T lymphocytes. A, 2 × 10^5 Jurkat cells or B, purified T cells (black lines) and IL-2-maintained T lymphoblasts (grey line) were stained with 10 μg/ml anti-CXCR4 Ab 12G5 or IgG2a isotype control ab (filled histograms), as described in Materials and Methods.](http://www.jimmunol.org/)

![FIGURE 2. SDF-1 stimulates accumulation of PtdIns(3,4,5)P_3 in Jurkat cells. A total of 1 × 10^7 ^32P-labeled Jurkat cells were stimulated at 37°C with A, various concentrations of SDF-1 for 1 min or with 5 × 10^5 CHO-B7.1 cells for 5 min, and B, 100 nM SDF-1 for the times indicated. Following stimulation, PtdIns(3,4,5)P_3 was extracted and deacylated, and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 were analyzed using HPLC, as described under Materials and Methods. The data are representative of at least four separate experiments. * Significantly different from control levels at p < 0.05; **, significantly different from control levels at p < 0.01 (two-way ANOVA with Dunnett’s t test).](http://www.jimmunol.org/)
FIGURE 3. SDF-1-stimulated PtdIns(3,4,5)P₃ accumulation is inhibited by SDF-1 peptide analogues and anti-CXCR4 mAb. A total of 1 × 10⁶ ³²P-labeled Jurkat cells were left untreated (shaded histobar) or treated as indicated with 40 µM of the SDF-1 peptide analogues (SDF-1 1–9, SDF-1 1–9 dimer, or SDF-1 1–9 [P2G] dimer), 10 µg/ml anti-CXCR4 Ab 12G5, or IgG2a isotype-matched control in the absence (open histobars) or presence (solid histobars) of 10 nM SDF-1. The SDF-1 peptide analogues were added in combination with SDF-1 for 1 min. The SDF-1 1–9 [P2G] dimer, anti-CXCR4 mAb 12G5, and isotype-matched IgG2a control were incubated for 15 min at 37°C before the addition of 10 nM SDF-1 for 1 min. Following stimulation, PtdIns(3,4,5)P₃ was extracted and deacylated, and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P₃ were analyzed using HPLC, as described under Materials and Methods. The data are representative of at least four separate experiments and are presented as the fold increase above the unstimulated control basal levels of PtdIns(3,4,5)P₃ (1619 ± 316 cpm).

levels than that observed for SDF-1 (Fig. 3). However, when these partial agonist peptides were added in combination with SDF-1, the resulting PtdIns(3,4,5)P₃ accumulation was reduced compared with that induced by SDF-1 alone (Fig. 3). The peptide antagonist SDF-1 1–9 [P2G] dimer and the anti-CXCR4 Ab 12G5 had no effect on basal levels of PtdIns(3,4,5)P₃, but did markedly inhibit accumulation of this lipid in response to 10 nM SDF-1 (Fig. 3).

We next investigated whether SDF-1 regulated PI 3-kinase activity in normal T lymphoblasts that have been previously maintained in IL-2 for 10 days. This treatment with IL-2 markedly down-regulates CXCR4 expression on T lymphoblasts compared with Jurkat and peripheral blood-derived T lymphocytes (Fig. 1, A and B). However, despite the low level of expression of CXCR4 on T lymphoblasts, SDF-1 could still elicit elevation of [Ca²⁺]i in these cells (Fig. 4A), thus both confirming previous observations (14) and demonstrating that CXCR4 stimulation can lead to biochemical events in this model. Pretreatment with the Gᵢ protein inhibitor pertussis toxin completely inhibited the elevation of [Ca²⁺]i in response to SDF-1 (Fig. 1, A and B). In marked contrast, although CXCR4 was expressed at much greater levels on Jurkat cells, 100 nM SDF-1 did not stimulate any detectable changes in [Ca²⁺]i, in Jurkat cells (data not shown). This suggests that the CXCR4 receptor is differentially coupled to biochemical signaling pathways in different T cell models, at least with respect to calcium mobilization. However, this differential signaling does not appear to extend to coupling to PI 3-kinase because accumulation of PtdIns(3,4,5)P₃ in response to SDF-1 was also observed in T lymphoblasts (Fig. 4B), the kinetics of which were similar to that observed for PtdIns(3,4,5)P₃ accumulation in Jurkat cells after SDF-1 treatment (Fig. 2B). Studies with PI 3-kinase inhibitors in other systems have demonstrated a requirement for D-3 phosphoinositide lipids for optimal receptor-stimulated calcium mobilization (57). However, pretreatment with the PI 3-kinase inhibitor wortmannin (58) had no effect on SDF-1-stimulated accumulation of [Ca²⁺]i in T lymphoblasts (Fig. 4A).

Effects of pertussis toxin or wortmannin pretreatment on SDF-1-stimulated PI 3-kinase activation

To further characterize the SDF-1-stimulated PI 3-kinase activity, we used the PI 3-kinase inhibitor wortmannin and the Gᵢ protein inhibitor pertussis toxin. Pretreatment for 10 min with wortmannin abrogated SDF-1-induced PtdIns(3,4,5)P₃ accumulation in both Jurkat cells and T lymphoblasts (Fig. 5, A and B). Similarly, pretreatment of Jurkat cells for 16 h with 100 ng/ml pertussis toxin completely abrogated the SDF-1-induced increase in
activity vs that present in immunoprecipitates derived from unstimulated cells. Accordingly, SDF-1 stimulated an increase in the in vitro activity of PI 3-kinase-γ that was extremely rapid and transient, because it was detectable 30 s after stimulation and had returned toward basal levels 5–10 min after SDF-1 treatment (Fig. 5C).

**SDF-1 stimulates PKB activation**

Having established that SDF-1 could strongly stimulate the activation of PI 3-kinase, we next examined the outcome of SDF-1 treatment on the activity of PKB, a known downstream effector of the PI 3-kinase-dependent signaling cascade (44). Hence, cell lysates derived from resting and SDF-1-stimulated cells were immunoblotted using a phosphospecific Ab that recognizes only the Ser473-phosphorylated, active form of PKB. Indeed, SDF-1 was shown to activate PKB within 30 s above the basal levels of PKB activity that were detectable under these conditions. Pretreatment of Jurkat cells for 16 h with 100 ng/ml pertussis toxin completely abrogated the SDF-1-stimulated PKB phosphorylation (Fig. 6). Similarly, 5-min pretreatment with the PI 3-kinase inhibitor wortmannin also inhibited SDF-1-stimulated PKB phosphorylation (Fig. 6). Basal levels of PKB activity were unaffected by either pertussis toxin or wortmannin (Fig. 6).

**Effects of PI 3-kinase inhibitors on SDF-1-induced chemotactic response**

Because chemokine receptor stimulation can have biological effects in the absence of measurable calcium mobilization (48), we investigated whether SDF-1 could stimulate chemotaxis of Jurkat cells and, if so, whether PI 3-kinase activation was involved. Indeed, SDF-1 stimulated the chemotaxis of Jurkat cells and freshly isolated peripheral blood-derived T lymphocytes in a bell-shaped, concentration-dependent manner that is characteristic of chemo-kine-dependent chemotaxis (Fig. 7, A and B) (48). The involvement of PI 3-kinase in this SDF-1-stimulated functional response was assessed by the use of the PI 3-kinase inhibitors wortmannin and LY294002. Jurkat cell and peripheral blood-derived T lymphocyte chemotaxis in response to SDF was inhibited by pretreatment of the cells with wortmannin (Fig. 7, A and B) and LY294002 (Fig. 7C). The IC₅₀ values for wortmannin and LY294002 inhibition of SDF-1-stimulated chemotaxis were 7 ± 4 nM and 1 ± 0.2 µM (n = 4). Pertussis toxin also inhibited the SDF-1-stimulated chemotaxis of Jurkat cells and peripheral blood-derived lymphocytes (Fig. 7, A and B).

**Effects of PI 3-kinase inhibitors on SDF-1-induced actin polymerization**

Rearrangement of the actin cytoskeleton is an early cellular response during chemotactic responses (59). Given the strong activation of a pertussis toxin-sensitive PI 3-kinase by SDF-1 and its apparent involvement in chemotaxis, we therefore investigated the effect of PI 3-kinase inhibitors on SDF-1-stimulated actin polymerization. We were unable to detect any SDF-1-stimulated changes in actin polymerization above the high basal levels observed in Jurkat cells (data not shown), even though SDF-1 stimulates chemotaxis of Jurkat cells. The reasons for this are unclear, but it is likely that levels of polymerized actin were so high as to prevent detection of any further effect of SDF-1 using the assay employed. We therefore used normal peripheral blood-derived T cells that exhibited much lower levels of basal actin polymerization. We were unable to detect any SDF-1-stimulated changes in actin polymerization above the high basal levels observed in Jurkat cells (data not shown), even though SDF-1 stimulates chemotaxis of Jurkat cells. The reasons for this are unclear, but it is likely that levels of polymerized actin were so high as to prevent detection of any further effect of SDF-1 using the assay employed. We therefore used normal peripheral blood-derived T cells that exhibited much lower levels of basal actin polymerization, and SDF-1 induced a marked concentration-dependent increase in actin polymerization in these cells (Fig. 8A), confirming previous observations (9). Moreover, the increase in filamentous actin was transient, occurring within 15 s and returning to basal

---

**FIGURE 5.** Effects of pertussis toxin and wortmannin on the SDF-1-induced accumulation of PtdIns(3,4,5)P₃. A total of 1 × 10⁷ [³²P]labeled Jurkat cells (A) and T lymphoblasts (B) were left unstimulated (open histobars) or were pretreated for 10 min with vehicle (solid histobars) or 100 nM wortmannin (hatched histobars). Alternatively, Jurkat cells (A) were pretreated with 100 ng/ml pertussis toxin (cross-hatched histobars) for 16 h before the [³²P]orthophosphate labeling of the cells, as described under Materials and Methods. After appropriate incubation with either pertussis toxin or wortmannin, 1 × 10⁷ cells were then stimulated at 37°C with 100 nM SDF-1 for the times indicated, and phospholipids were extracted and deacylated, and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P₃ were analyzed by HPLC. The data are representative of at least four separate experiments and are presented as the fold increase above the unstimulated control basal levels (open histobars) of PtdIns(3,4,5)P₃ (A, 3232 ± 343 cpm; B, 950 ± 196). C, A total of 1 × 10⁷ Jurkat cells were stimulated at 37°C for various times with 100 nM SDF-1. Cells were lysed and lysates were subjected to immunoprecipitation with an anti-PI 3-kinase-γ Ab. The washed immunoprecipitates were analyzed for PtdIns kinase activity, as described under Materials and Methods. Lipids were detected by exposure to film at −70°C. The data are representative of at least three separate experiments.

PtdIns(3,4,5)P₃ (Fig. 5A), suggesting that the accumulations of PtdIns(3,4,5)P₃ following SDF-1 treatment appear to involve a G_{i} protein-mediated mechanism. To verify this, we determined whether immunoprecipitates of PI 3-kinase-γ derived from SDF-1-stimulated Jurkat cells exhibited enhanced in vitro lipid kinase activity vs that present in immunoprecipitates derived from unstimulated cells. Accordingly, SDF-1 stimulated an increase in the in vitro activity of PI 3-kinase-γ that was extremely rapid and transient, because it was detectable 30 s after stimulation and had returned toward basal levels 5–10 min after SDF-1 treatment (Fig. 5C).
levels within 30 min (Fig. 8B). Pretreatment with either wortmannin or LY294002 partially inhibited actin polymerization by 50 ± 3% and 58 ± 6%, respectively (Fig. 8C). In contrast, pertussis toxin pretreatment completely abrogated actin polymerization in response to SDF-1 treatment of the cells (Fig. 8C).

PI 3-kinase inhibitors prevent SDF-1-stimulated ERK1/2 phosphorylation

Having established that SDF-1 could strongly stimulate a pertussis toxin-sensitive PI 3-kinase, we next examined the outcome of PI 3-kinase inhibitors on SDF-1-stimulated ERK1/2 MAP kinase activation because the pertussis toxin-sensitive PI 3-kinase-γ has been demonstrated to mediate Gβγ-dependent regulation of the MAP kinase signaling pathway in other systems (60, 61). Hence, cell lysates derived from control unstimulated or SDF-1-stimulated Jurkat cells were immunoblotted using a phosphospecific Ab to the phosphorylated active forms of ERK1/2. Indeed, SDF-1 was shown to activate ERK1/2 within 30 s (Fig. 9A). Pretreatment of Jurkat cells for 10 min with wortmannin inhibited the SDF-1-stimulated ERK phosphorylation in a concentration-dependent manner (Fig. 9, B and D). The activation of ERK1/2 in response to SDF-1 was also inhibited by 16-h pretreatment with pertussis toxin (Fig. 9C). Blots were routinely stripped and reprobed with anti-ERK1/2 Ab to verify equal loading and efficiency of protein transfer (Fig. 9A–D).

Inhibition of MEK inhibits SDF-1-stimulated chemotaxis

Given that PI 3-kinase activation is required for ERK1/2 activation as well as chemotaxis in response to SDF-1, we investigated whether the chemotactic response of peripheral blood-derived T lymphocytes was also dependent on ERK1/2 activation using the MEK inhibitor PD098059 (62). Indeed, peripheral blood-derived T cell chemotaxis, in response to a concentration of SDF-1 sufficient to elicit optimal chemotaxis (10 nM), was attenuated by pretreatment of the cells with PD098059 (Fig. 10). Although PD098059 inhibition of SDF-1-stimulated chemotaxis was concentration dependent, the highest concentration (10 μM) afforded only partial inhibition to 58 ± 7% of control migration (n = 4).

Discussion

This study has demonstrated that SDF-1 and its peptide analogues induce a concentration- and time-dependent accumulation of PtdIns(3,4,5)P₃ in Jurkat cells. This SDF-1-stimulated generation of D-3 phosphoinositide lipids was inhibited by pretreatment of the cells with either an SDF-1 peptide antagonist, an anti-CXCR4 Ab, and PI 3-kinase inhibitors, or the G protein inhibitor pertussis toxin. An interesting observation from these studies was that SDF-1 was unable to stimulate increases in [Ca²⁺] in Jurkat cells, although these cells still elicited a chemotactic response to SDF-1. This further supports the notion that chemokine receptor stimulation can have biological effects in the absence of measurable calcium mobilization (48). Moreover, it would appear that activation of the PI 3-kinase-dependent signaling cascade plays a pivotal role in chemotaxis, given that PI 3-kinase inhibitors prevent chemotaxis of Jurkat cells and peripheral blood-derived T lymphocytes. These results provide the first demonstration that SDF-1 stimulates rapid and large accumulations of PtdIns(3,4,5)P₃ involving a G protein-mediated mechanism.

The elevation of PtdIns(3,4,5)P₃ observed in response to SDF-1 may be the result of activation of more than one PI 3-kinase (e.g., the p85/p110 PI 3-kinase and PI 3-kinase-γ). However, the accumulation of PtdIns(3,4,5)P₃ in Jurkat cells stimulated by SDF-1 could be completely inhibited by pretreatment with pertussis toxin, strongly indicating that D-3 phosphoinositide lipid accumulation occurs via a G protein-coupled PI 3-kinase. To date, the only characterized G protein-coupled PI 3-kinase is the class IA PI 3-kinase (32). Previous studies using different cell models have reported that SDF-1 stimulation induces a lipid kinase activity to coassociate with antiphosphotyrosine immunoprecipitates, implying the activation of the class IA p85/p110 heterodimer, although this was not formally demonstrated (31). Other studies have reported an increase in PI 3-kinase activity associated with antiphosphotyrosine immunoprecipitates after activation of G protein-coupled receptors (35, 48, 49). Certainly, SDF-1 can induce the protein tyrosine phosphorylation of a number of substrates (28–31), while other G protein-coupled receptors have also been shown to stimulate protein tyrosine kinases after appropriate stimulation with bombesin and vasopressin (63) or monocyte-chemoattractant protein-1 (49). Because synergistic activation of the p85/p110 PI 3-kinase by tyrosine-phosphorylated peptides and Gβγ subunits of GTP-binding proteins has been reported (36, 64), it is possible that the p85/p110 heterodimer may contribute to the accumulation of PtdIns(3,4,5)P₃ observed after stimulation with SDF-1. However, it seems unlikely that the p85/p110 heterodimeric PI 3-kinase makes any contribution to SDF-1-stimulated PtdIns(3,4,5)P₃ accumulation, because this response is completely abrogated by pertussis toxin pretreatment, while the protein tyrosine kinase inhibitor herbimycin A had no effect on the PtdIns(3,4,5)P₃ accumulation (our unpublished observations). Hence, the different levels of PtdIns(3,4,5)P₃ accumulation stimulated by SDF-1 and CD28 may be simply explained by the fact that they stimulate different subclasses of PI 3-kinase, namely PI 3-kinase-γ and p85/p110, respectively.
Studies with PI 3-kinase inhibitors in other systems have demonstrated a requirement for D-3 phosphoinositides in the activation of phospholipase C-\(\gamma\) and hence for optimal calcium mobilization in response to ligation of the B cell Ag receptor (57, 65). Such a mechanism is thought to involve direct interaction of D-3 phosphoinositide lipids with the tandem Src homology 2 domains and/or the amino-terminal PH domains of phospholipase C-\(\gamma\). In addition, the D-3 phosphoinositides can interact with the PH domains of the Tec family of protein tyrosine kinases, thereby influencing their membrane targeting and activation, which in turn influences phospholipase C-\(\gamma\) activation. However, CXCR4 appears to be coupled to the pertussis toxin-sensitive phospholipase C\(\beta\) and it is unlikely that phospholipase C\(\gamma\) is activated by CXCR4. The G protein-coupled \(\beta\) isoforms of phospholipase C also contain a PH domain that can potentially interact with the D-3 phosphoinositides formed in response to SDF-1 and hence facilitate optimal calcium mobilization. However, this seems an unlikely event given

![FIGURE 7](image_url)

**FIGURE 7.** Effects of PI 3-kinase inhibitors on the SDF-1-induced chemotactic response in Jurkat and normal T lymphocytes. A total of \(2 \times 10^5\) Jurkat cells (A) and normal T lymphocytes (B) were incubated with vehicle (■) wortmannin (100 nM for 10 min, ●), or pertussis toxin (100 ng/ml for 16 h, ▲) at 37°C and then incubated with increasing concentrations of SDF-1 (1–100 nM) in a 96-well chemotaxis chamber at 37°C for 2 h. C. A total of \(2 \times 10^5\) Jurkat (■) and peripheral blood-derived T lymphocytes (●) were incubated with LY294002 for 10 min at 37°C and then incubated with SDF-1 (10 nM) in a 96-well chemotaxis chamber at 37°C for 2 h. Cell migration (A, B, and C) was assessed using Cell Titer 96 AQ solution, as described under Materials and Methods. Results are expressed as a mean chemotactic index (±SEM), which is the ratio of OD readings of the stimulated samples against the OD readings of the control samples incubated with medium alone, from quadruplicate wells. The data are representative of at least three separate experiments.

![FIGURE 8](image_url)

**FIGURE 8.** Effects of wortmannin on the SDF-1-induced actin polymerization. Peripheral blood-derived T lymphocytes (\(2 \times 10^6\) cells/0.5 ml) were stimulated with SDF-1 (1–1000 nM) for 1 min (A) or with 100 nM SDF-1 for the times indicated (B). C. Alternatively, T lymphocytes were stimulated with 100 nM SDF-1 for 1 min in the absence (black histobar) or presence of 100 nM wortmannin (hatched histobars), 10 \(\mu\)M LY 294002 (lined histobars), or 100 ng/ml pertussis toxin (cross-hatched histobars). Cells were incubated at 37°C for 10 min with wortmannin and LY294002 and 16 h with pertussis toxin. Actin polymerization was assessed as described in Materials and Methods and is expressed as a percentage increase above the resting control levels of polymerized actin.
that we were unable to detect any inhibitory effect of the PI 3-kinase inhibitor wortmannin on elevation of \([\text{Ca}^{2+}]\) in T lymphoblasts in response to SDF-1 stimulation.

The SDF-1-induced activation of a wortmannin-sensitive PI 3-kinase appears to be an important signal required for SDF-stimulated biochemical events such as ERK1/2 and PKB phosphorylation. Although SDF-1 can couple to these distinct signaling pathways that can mediate cell survival, growth, migration, and transcriptional activation, it is unable to support IL-2 production and T cell proliferation either alone or in combination with anti-CD3 or anti-CD28 Abs (unpublished observations). One possibility is that SDF-1 may regulate the threshold for T cell activation. Indeed, some studies have demonstrated that SDF-1 can exert inhibitory effects on critical components of the TCR signaling cascade such as reduced tyrosine phosphorylation of ZAP-70, SLP-76, and linker for activation of T cells (66), while PI 3-kinase has been proposed to play a negative role in TCR function (67). Our observation that SDF-1 activates PKB also fits well with the previous demonstration that PKB is a downstream effector of PI 3-kinase-\(\gamma\) (68). Indeed, several other \(G\) protein-coupled receptors, including those activated by the chemokines RANTES and IL-8, have been shown to activate PKB in a PI 3-kinase-dependent manner, although the functional significance of these observations has not been determined (69, 70). PKB is a key mediator of growth factor-induced cell survival and protection against c-Myc-induced cell death (71–73). However, we have demonstrated that pretreatment of Jurkat cells with SDF-1 is not sufficient to protect against Fas-induced Jurkat cell death (71–73). Nevertheless, activation of PKB by SDF-1 is hard to reconcile with evidence implicating SDF-1 and CXCR4 with the promotion of cell death in various systems (74–76).

Several studies have recently reported that SDF-1 stimulates phosphorylation of MEK-1 and ERK1/2 in leukemic T cell lines, T cell clones, and a pre-B cell lymphoma cell line (28–31). Our data indicate that PI 3-kinase inhibitors prevent SDF-1-stimulated activation of ERK1/2, implying an upstream requirement for PI 3-kinase activation. These observations correlate well with observations that PI 3-kinase-\(\gamma\) has been demonstrated to mediate \(G\beta\gamma\)-dependent regulation of both the ERK1/2 MAP kinase and PKB signaling pathway in other systems (60, 61, 68). Moreover, the MEK inhibitor PD098059 partially inhibits SDF-1-stimulated chemotaxis, suggesting that ERK1/2 activation may be involved at least in part, as a downstream effector of a PI 3-kinase-regulated signaling cascade that culminates in a chemotactic response. This would correlate with previous observations indicating a role for MAP kinases in amoeboid chemotaxis in response to cAMP and fibroblast chemotaxis in response to fibronectin (77–79). However, it should be emphasized that chemotaxis of neutrophils in response to the related chemokine IL-8 or fMLP has been reported to be independent of ERK1/2 (47, 80, 81). Hence, our observation that ERK1/2 activation is involved at least partially in SDF-1-stimulated chemotaxis in T cells may reflect differences between cell types and/or chemoattractants with respect to the biochemical pathways that facilitate chemotactic responses.

The role of SDF-1-stimulated biochemical signals in T cell activation remains unclear, but it seems that SDF-1-stimulated activation of \(G_i\) protein-coupled PI 3-kinase plays a pivotal role in...
chemokinesis, given that PI 3-kinase inhibitors prevent chemokinesis of Jurkat cells and peripheral blood-derived T lymphocytes. This correlates well with previous studies that have indicated that PI 3-kinase and its metabolic products play an important role in signaling pathways mediated by chemokinesis (47–49, 80, 81). Moreover, PI 3-kinase-γ has been shown to play an important role in regulating reorganization of the actin cytoskeleton (82), a process thought to be a prerequisite for cell movement (59). However, it is interesting to note that SDF-1 induces CXCR4 coupling to both Gαi (pertussis toxin-sensitive) (9, 19) and Gαq (pertussis toxin-insensitive) (9, 19, 20, 83) family members of G proteins, so it is possible that CXCR4 uses more than one G protein subunit and may initiate signaling pathways that are independent of PI 3-kinase(s). Another possibility is that there may be more than one receptor for SDF-1 and these receptors may be differentially coupled to signaling pathways and functional events. Indeed, while SDF-1−/− and CXCR4−/− mice have similar phenotypes relating to B cell development, they may not be identical in other respects (24–27) and a splice variant of CXCR4 has indeed recently been identified (84). It is also interesting to note that the optimal changes in actin polymerization were observed 30 to 1 min after SDF-1 stimulation, but chemokinesis was not measured 2 h after stimulation. Therefore, a final possibility to explain the different sensitivities of SDF-1-stimulated chemokinesis and actin polymerization to PI 3-kinase inhibitors is that while the observed changes in polymerized actin may well be a representation of initiation of motile response, these changes may have little to do with the subcellular contractile machinery for sensing chemotactic gradients and facilitating ordered and coordinated cell migration. This investigation has demonstrated that SDF-1 activates a pertussis toxin-sensitive PI 3-kinase that appears necessary for the activation of PKB and ERK in response to SDF-1. Although the role of PKB in SDF-1 functional responses is not understood, it seems that PI 3-kinase-dependent ERK activation is required for SDF-1-stimulated chemokinesis. Interestingly, other studies have reported that G protein-coupled receptors can activate multiple PI 3-kinase effectors such as Rac and PKB (82, 85). However, while cytoskeletal reorganization and lamellipodium formation are PI 3-kinase-mediated events, they occur independently of PKB (82, 85). Use of PI 3-kinase inhibitors such as wortmannin and LY294002 does not distinguish between the lipid or protein kinase activities of PI 3-kinases. Given that PKB activation is dependent on D-3 phosphoinositide products of PI 3-kinase-γ, while MAP kinase activation is mediated by the protein kinase activity of PI 3-kinase-γ in other systems (68), it will be an important aim of future studies to ascertain whether it is the lipid or protein kinase activity of PI 3-kinase responsible for mediating functional effects of SDF-1.

References


