Signal Transduction by CXC Chemokine Receptor 4: Stromal Cell–derived Factor 1 Stimulates Prolonged Protein Kinase B and Extracellular Signal–regulated Kinase 2 Activation in T Lymphocytes

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Abstract

We report that stromal cell–derived factor (SDF)-1 has the remarkable capacity to induce sustained signaling through CXC chemokine receptor 4 (CXCR4). In contrast to other chemokines, such as monocyte chemotactic protein 1 (CC chemokine receptor 2 [CCR2]), macrophage inflammatory protein 1β (CCR5), liver and activation-regulated chemokine (LARC [CCR6]), Epstein-Barr virus–induced molecule 1 ligand chemokine (ELC [CCR7]), and IP10 (CXCR3), SDF-1 stimulates the prolonged activation of protein kinase B and extracellular signal–regulated kinase (ERK)-2. Activation of protein kinase B is reversed by displacement of SDF-1 from CXCR4 or inhibition of phosphatidylinositol 3-kinase. Although increasing concentrations of SDF-1 enhance CXCR4 internalization, kinase activation is prolonged. In addition, restimulation yields >60% of initial protein kinase B activity, indicating that the remaining receptors are not desensitized. Furthermore, activation is prolonged by inhibiting SDF-1 degradation. The sustained activation of cell survival and mitogenic pathways may account for the unique role of SDF-1 and CXCR4 in embryogenesis and lymphopoiesis.

Key words: CXCR4 • SDF-1 • signal transduction • protein kinase B • PI 3-kinase

Introduction

Chemokines constitute the largest family of cytokines, with more than 50 distinct members acting on at least 16 different receptors (1, 2). Initially, chemokines were characterized as inflammatory mediators. It is now becoming clear that the chemokine system is involved in many physiological and pathological processes, such as inflammation, tumorigenesis, hematopoiesis, development, embryogenesis, and HIV infection (1, 3–5). All chemokine receptors belong to the same class of seven transmembrane domain receptors and are associated with heterotrimeric G_i proteins. Despite their structural similarity and the coupling to the same type of G protein, they can activate specific signal transduction pathways leading to diverse responses. However, the association of the receptors with distinct signal transduction pathways is poorly understood.

Expansion of blood-derived T lymphocytes in the presence of IL-2 induces the expression of several chemokine receptors that can mediate various functional responses (6). These cells can therefore be used for the study of molecular mechanisms of signal transduction initiated by chemokine receptors. Many chemokines bind to more than one receptor, and the receptors generally bind more than one chemokine (1). Stromal cell–derived factor (SDF)[1]-1, by

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[1]*Abbreviations used in this paper:* CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; ELC, EBV-induced molecule 1 ligand chemokine; ERK, extracellular signal–regulated kinase; LARC, liver and activation-regulated chemokine; MAP, mitogen-activated protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PI 3-kinase, phosphatidylinositol 3-kinase; RANTES, regulated on activation, normal T cell expressed and secreted protein; SDF, stromal cell–derived factor.

contrast, is the unique ligand for CXC chemokine receptor 4 (CXCR4). Targeted gene disruption of SDF-1 or CXCR4 in mice is lethal, and morphological analysis of embryos revealed similar phenotypes in both deficiencies, suggesting that SDF-1 interacts exclusively with CXCR4 and vice versa (7–9). The experiments further demonstrated that SDF-1 is not a classical inflammatory chemokine but is essential for normal embryogenesis since organogenesis, lymphopoiesis, vascularization, and the development of the central nervous system were impaired in the knockout animals. CXCR4 acts as coreceptor for infection with X4-tropic HIV strains (10). SDF-1 protects against HIV-1 infection by competing with gp120 for binding to CXCR4 and by downregulation of the receptor from the cell surface (11).

Signal transduction by chemokine receptors leads to the activation of G proteins and phospholipase C and the elevation of cytosolic free calcium (2). We and others have shown that stimulation of chemokine receptors results in the transient activation of the mitogen-activated protein (MAP) kinase extracellular signal–regulated kinase (ERK)-2 (12–14). Activation of ERK-2 is Ras dependent, and prolonged activation causes its nuclear translocation and activation of transcription (15). Recently, it was shown that chemokines stimulate phosphatidylinositol 3-kinase (PI 3-kinase) leading to the formation of phosphatidyl 3,4,5,-triphosphate (PIP_3; 16, 17) and the activation of protein kinase B (also termed Akt or RAC-PK) (18). Attenuation of PI 3-kinase with the specific inhibitor wortmannin (19) abrogates protein kinase B activation (20). Thus, PI 3-kinase activity is necessary and sufficient to stimulate protein kinase B (21). Activation of protein kinase B is accomplished by binding of its pleckstrin homology (PH) domain to 3-phosphoinositides and the phosphorylation of two critical residues (Thr^{308} and Ser^{473}) by phosphoinositide-dependent kinase(s) (22). Activated protein kinase B promotes cell survival by phosphorylating and inactivating several apoptosis-mediating proteins such as BAD (23), caspase-9 (24), and a forkhead transcription factor (25, 26).

In this study, we demonstrate that SDF-1 has the remarkable capacity to promote prolonged signaling through CXCR4 in T lymphocytes. Although other chemokines induce a transient activation of protein kinase B and ERK-2, SDF-1 stimulates a markedly enhanced and prolonged activation of the two signal transduction pathways. Sustained protein kinase B activation is shown to depend on active CXCR4 persisting at the cell surface, interaction with G_i proteins, and the continuous activation of PI 3-kinase. Furthermore, we found that the subsistence of sustained kinase activation is more prominent in the presence of inhibitors of dipeptidyl peptidase CD26 and is regulated by the internalization of CXCR4.

Materials and Methods

Materials. Cell culture media and supplements were obtained from GIBCO BRL. Monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, liver and activation-regulated chemokine (LARC), EBV-induced molecule 1 ligand chemokine (ELC), IP10, SDF-1, and SDF-1P2G (27) were chemically synthesized. Human recombinant IL-2 was provided by Dr. A. Lanzavecchia (Basel Institute of Immunology, Basel, Switzerland). Crosstide (28) and 17-hydroxywortmannin (29) were gifts from Dr. B.A. Hemmings (Friedrich Miescher-Institute, Basel, Switzerland) and Dr. T. Payne (Novartis, Basel, Switzerland), respectively.

Antibodies. Alkaline phosphatase– and horseradish peroxidase–conjugated goat anti–rabbit or anti–mouse IgG were from Bio-Rad Laboratories; anti–ERK-2 was from Santa Cruz Biotechnology, Inc., and anti-AKT/protein kinase B and phospho-AKT/protein kinase B (Ser^{473}) antibodies were from New England Biolabs, Inc. RPE-conjugated goat anti–mouse IgG was from Dako; anti–MAP kinase (activated, diphosphorylated ERK-1 and ERK-2) and mouse isotype controls were from Sigma-Aldrich. The murine mAb to CXCR4 (6H8, IgG_1) was provided by Dr. Fernando Arenzana-Seisdedos (Pasteur Institute, Paris, France). The anti-CXCR4 mAb 12G5 (IgG_{2a}) was a gift from Dr. J. Hoxie (University of Pennsylvania Medical Center, Philadelphia, PA), and the anti-CCR5 mAb 5C7 (IgG_{2a}) was provided by LeukoSite, Inc. (Cambridge, MA).

Cell Preparation and Culture. Human PBMCs were isolated from donor blood (Swiss Red Cross Laboratory) by Ficoll-Paque gradient centrifugation. PBLs were obtained by removing monocytes by adherence or by Percoll gradient centrifugation (30). Lymphocytes were cultured in RPMI supplemented with 1% glutamine, nonessential amino acids, sodium pyruvate and kanamycin, 50 μM 2-ME, 5% human serum (Swiss Red Cross Laboratory), and human recombinant IL-2 (200 U/ml). The cell density was kept between 1 and 3×10^6 cells/ml by diluting with medium containing IL-2. After 6 d, >90% of the cells were $CD3^+$ and they were used after 9–12 d of IL-2 treatment (6).

Cell Stimulation. If not stated otherwise, IL-2–expanded T cells were serum-starved overnight in medium supplemented with 200 U/ml IL-2. The cells were washed twice and resuspended in HBSS containing 20 mM Hepes, pH 7.4 (2×10^7 cells/ml). Aliquots of 2×10^6 cells for immunoblotting and 6×10^6 cells for immunoprecipitation were incubated for 10 min at 37°C and then stimulated with the appropriate agonist. For Western blot analysis, the incubations were terminated by the addition of TCA. The incubation of samples for immunoprecipitation was stopped by the addition of double concentrated ice-cold lysis buffer (50 mM Tris-Cl, pH 7.5, 1% NP-40, 120 mM NaCl, 1 mM EDTA, 25 mM NaF, 40 mM β-glycerophosphate, 0.1 mM sodium vanadate, 0.5 mM PMSF, 1 mM benzamidine, and 1 μM Microcystin-LR; Alexis Corp.), and the samples were immediately frozen in liquid nitrogen.

Immunoprecipitation and In Vitro Kinase Assay. Frozen lysates were thawed on ice, centrifuged for 10 min at 12,000 *g* at 4°C, and precleared with protein A–Sepharose. Protein kinase B was immunoprecipitated from 5×10^6 cell equivalents for 3 h at 4°C with anti-AKT/protein kinase B antibody. Immunoprecipitates were washed once with lysis buffer containing 0.5 M NaCl, once with lysis buffer, and twice with kinase buffer (50 mM Tris-Cl, pH 7.5, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM benzamidine, and 1 μM Microcystin-LR). Kinase assays were performed for 2 h as described previously using Crosstide (GRPRTSS-FAEG) as substrate (28). The immunoprecipitated protein kinase B content was analyzed by Western blotting.

Western Blot Analysis. Immunoprecipitated or total protein samples were resolved on 11% SDS-PAGE and transferred to Immobilon-P membranes. The membranes were incubated with the

corresponding antibody overnight. Enhanced chemiluminescence was used for detection of horseradish peroxidase–conjugated secondary antibodies. Membranes were subsequently stripped using 0.2 M NaOH for 5 min, washed, and reprobed with the appropriate antibodies. For ERK-2 detection, alkaline phosphatase–conjugated antibodies were used.

Phosphoinositide Determination. Approximately 1.2×10^8 serum-starved, IL-2–expanded T cells were loaded with 0.3 mCi/ml [^{32}P]orthophosphate (Amersham Pharmacia Biotech) in 10 ml phosphate-free RPMI supplemented with 20 mM Hepes, pH 7.3, for 2 h at 37°C. The cells were washed twice in RPMI supplemented with 20 mM Hepes, pH 7.3, and resuspended at 6.5×10^7 cells/ml in the same medium. Aliquots of 150 μl were treated with chemokine, antagonist, or wortmannin in a final volume of 170 μl. Reactions were quenched by the addition of 640 μl CH_3Cl/MeOH (1:2), and lipids extracted were analyzed by HPLC as described (31).

Calcium. The intracellular free calcium concentration was determined as described previously (32).

Flow Cytometry. PBMCs (5×10^6/ml) or serum-starved IL-2–expanded T cells (2×10^7/ml) were incubated at 37°C in RPMI supplemented with 10% FCS in the presence or absence of SDF-1 for various times. Before staining with antireceptor antibodies, cell-bound chemokine was removed by an acidic glycine wash (11). Single-color analysis was performed with mouse mAbs against CCR5, CXCR4, or isotype-matched control IgG, followed by PE-conjugated goat anti–mouse IgG. For two-color analysis, the cells were first stained for the chemokine receptor, then saturated with 10% mouse serum before addition of FITC-conjugated mouse anti-CD3 antibodies. Cell-associated fluorescence was analyzed with a FACScan™ flow cytometer (Becton Dickinson).

Results

SDF-1 Stimulates Prolonged Protein Kinase B and ERK-2 Activation. Human T cells cultured for 9–12 d in the presence of IL-2 exhibit similar chemotactic responsiveness to several CXC and CC chemokines (33) and were therefore used to compare the signaling properties of different chemokine receptors. Under the experimental conditions used, each chemokine binds only to one receptor and induces receptor-specific signal transduction (33). SDF-1 binds to CXCR4 (32), MCP-1 to CCR2 (34), MIP-1β to CCR5 (35, 36), LARC to CCR6 (37), ELC to CCR7 (38), and IP10 to CXCR3 (39). As downstream effector, we investigated the activation of protein kinase B. To minimize basal activity of the kinase, the cells were starved overnight before treatment with the chemokines. Activation of protein kinase B correlates with its phosphorylation at two residues, Thr^{308} and Ser^{473} (22). We used an antibody that reacts specifically with the phosphorylated Ser^{473} located at the COOH terminus to determine chemokine-stimulated activation of protein kinase B. Fig. 1 A (left) shows a typical Western blot of whole cell lysates separated by SDS-PAGE. Except for the CCR6 ligand, LARC, all chemokines induced a rapid phosphorylation of protein kinase B. MCP-1, MIP-1β, and IP10 stimulated a transient activation that peaked at ~1 min and ceased within 2–5 min. The response to ELC was somewhat more protracted, lasting for up to 10–20 min (depending on the cell batch used). In contrast, SDF-1 stimulated a strong and markedly prolonged activation of protein kinase B that was detectable for up to 90 min. Total protein kinase B was determined by stripping and reprobing the blots with an antibody reacting with both the phosphorylated and nonphosphorylated enzyme (Fig. 1 A, right). Enzyme activity was measured in antiphospho-Ser^{473} immunoprecipitates using Crosstide as specific peptide substrate (28). The results confirmed that SDF-1 stimulates a protracted activation of protein kinase B that persists for at least 40 min (Fig. 1 B). Maximum kinase activity was consistently observed ~1 min after stimulation, whereas the extent varied between cells from different donors (10–20-fold activation). On Western blots, the strong initial stimulation appears less pronounced due to the poor linearity of the enhanced chemiluminescence detection method.

We next compared protein kinase B activity in immunoprecipitates obtained from cells stimulated with various chemokines (Fig. 1 C). MIP-1β– and MCP-1–induced kinase activity returned to baseline values within 5 min even at chemokine concentrations as high as 1 μM. Consistent with the Western blot analysis, ELC and SDF-1–elicited enzyme activity was more protracted. However, the most pronounced activation was again obtained with SDF-1. The stimulatory capacity of SDF-1 was not restricted to IL-2–expanded T cells. Similar levels of protein kinase B activation were obtained with freshly isolated CXCR4$^+$ PBLs, the human T cell lines CEMx174 and J.Jahn, the monocytic cell line GM-1, and HeLa cells. In all cases, SDF-1 induced a strong protein kinase B activation that lasted for at least 40 min. Pretreatment of the cells with *Bordetella pertussis* toxin abrogated the response to SDF-1, indicating that protein kinase B activation depends on coupling of CXCR4 to a heterotrimeric G_i protein.

Regulated on activation, normal T cell expressed and secreted protein (RANTES) is a potent activator of T cells and binds to two chemokine receptors, CCR1 and CCR5 (6, 40). Stimulation of IL-2–expanded T cells with RANTES resulted in a protein kinase B activation that was comparable to that induced by MCP-1 or MIP-1β, suggesting that CCR1 possesses similar stimulatory capacity as other receptors for inflammatory chemokine.

The prolonged activation of protein kinase B elicited by SDF-1 was unexpected and appeared to be a distinctive characteristic of the G_i protein–coupled receptor CXCR4. Therefore, we investigated whether SDF-1 causes protracted activation of other intracellular signal transduction pathways. Previous studies demonstrated that the MAP kinase kinase cascade leading to the activation of ERK-2 is regulated independently of protein kinase B (28), and chemokines were shown to stimulate ERK-2 activity in Jurkat cells (12). Using the same panel of chemokines, we compared the activation of ERK-2 in T cells. Fig. 1 D represents a Western blot analysis of ERK-2 activation performed with the same lysates as used in Fig. 1 A. Stimulation of ERK-2 results in the retarded electrophoretic mobility of the threonine- and tyrosine-phosphorylated enzyme that is detected by a specific antibody reacting with both resting and activated enzyme. Fig. 1 D shows that the

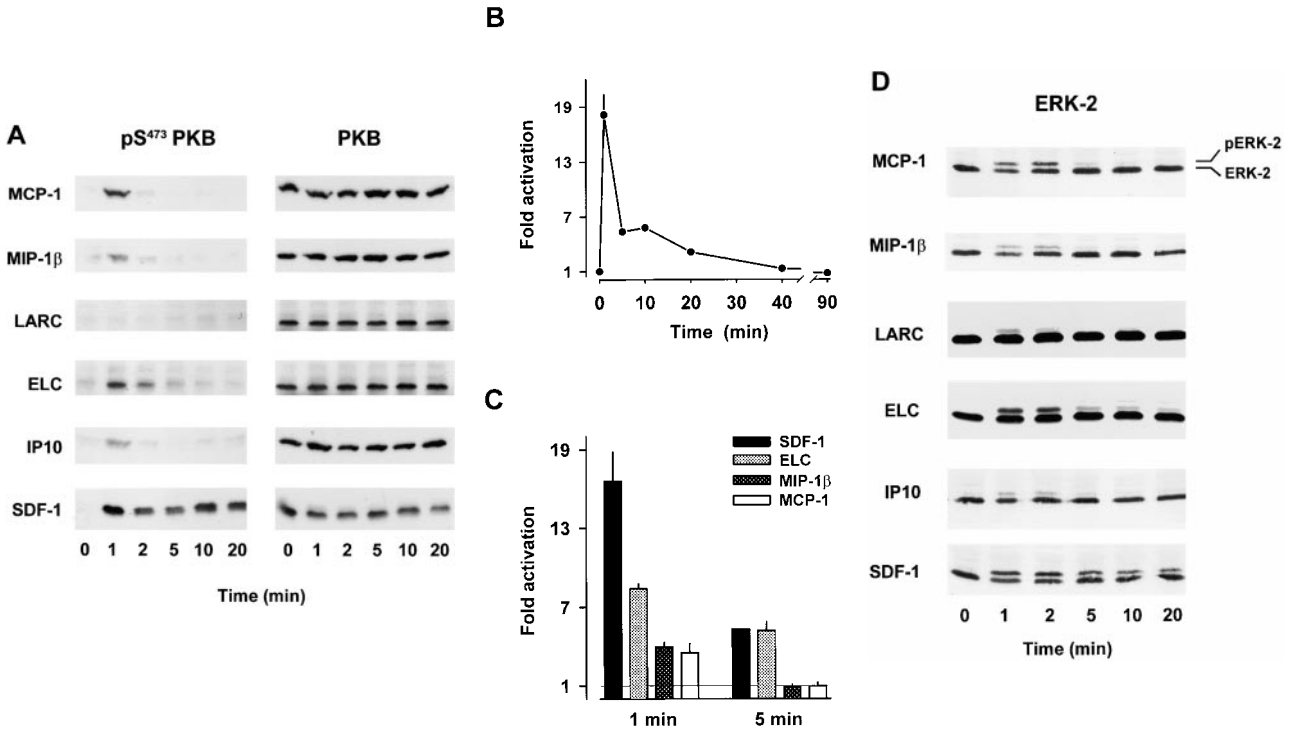

Figure 1. Chemokine-stimulated activation of protein kinase B and ERK-2 in T cells. (A) Activation of protein kinase B determined by Western blot analysis. T cells were stimulated with 200 nM MCP-1, MIP-1β, LARC, ELC, IP10, or SDF-1 for the indicated times. Whole cell lysates were separated on SDS-PAGE and activated protein kinase B was detected with an antibody reacting with phosphorylated Ser^{473} (pS^{473}PKB, left). To confirm equal loading of protein kinase B, blots reprobed with an antibody reacting with both activated and nonactivated protein kinase B (PKB, right). (B) Time course of protein kinase B activation. Enzyme activity was determined in immunoprecipitates from lysates of cells stimulated with 100 nM SDF-1. Kinase assays were performed in duplicate, and the results are presented as fold activation (mean values). (C) Comparison of protein kinase B activity in immunoprecipitates from cells stimulated for 1 or 5 min with 100 nM SDF-1, ELC, MCP-1, or MIP-1β. (D) Western blot analysis of ERK-2 activation. Cells were stimulated with 200 nM MCP-1, MIP-1β, LARC, ELC, IP10, or SDF-1 for the indicated times and whole cells lysates were separated by SDS-PAGE. Activated ERK-2 (pERK-2) migrates with a slower electrophoretic mobility than nonactivated ERK-2 (ERK-2). Representative results obtained with cells from single donors are shown. Independent experiments with at least four more batches of cells from different donors gave similar results.

most prominent and long-lasting effect was again obtained with SDF-1. With 100 nM SDF-1, phosphorylated ERK-2 was detectable for up to 90 min (see Fig. 4). The extent did not vary significantly between cell batches. The overall responses resembled the pattern obtained for protein kinase B stimulation. MCP-1, MIP-1β, and IP10 induced a transient activation of ERK-2, whereas the response to ELC was more protracted. LARC, which did not affect protein kinase B, showed a weak activation of ERK-2. Similar results were obtained when the blots were developed with a specific antibody detecting diphosphorylated ERK-1 and ERK-2.

Testing the chemokine concentration dependence of protein kinase B and ERK-2 activation revealed expected dose–response curves. Marginal activation of both kinases was obtained with 1 nM of chemokine and maximum activation was obtained with 100 nM (see Fig. 4, and data not shown). The finding is in good agreement with the reported affinities of the chemokines for their respective receptors (33) and confirms previous observations obtained with other chemokines (18).

Effect of the SDF-1 Antagonist SDF-1P2G on Protein Kinase B Activation. By substituting Pro^2 with glycine in the NH_2 terminus of SDF-1 (K[P→G]VSLSYRC…) a potent receptor antagonist, SDF-1P2G, is obtained that binds with similar high affinity as SDF-1 to CXCR4, i.e., 9 nM compared with 3–4 nM for SDF-1 (27). SDF-1P2G does not induce signal transduction or receptor downregulation even at 10 μM (not shown), and $CD4^+CXCR4^+$ cells are only marginally protected against infection with X4-tropic HIV strains (27). We used the antagonist to elucidate signal transduction mechanisms that are responsible for the prolonged stimulatory capacity of CXCR4. Fig. 2 A demonstrates that pretreatment of T cells with 10 μM SDF-1P2G completely inhibits SDF-1–stimulated protein kinase B activation. Moreover, SDF-1P2G did not induce activation of protein kinase B (Fig. 2 A) or ERK-2 (not shown). To test whether the antagonist can terminate protein kinase B activation if added after SDF-1, the cells were first stimulated with 100 nM SDF-1 for 5 or 10 min and then treated with 10 μM SDF-1P2G for 5 min. Fig. 2 B shows that the subsequent addition of SDF-1P2G leads to a rapid dephosphorylation of protein kinase B. Similar results were obtained when protein kinase B activity was determined in immunoprecipitates (Fig. 2 C). Stimulation with 100 nM SDF-1 for 10 min resulted in a sixfold increase in protein

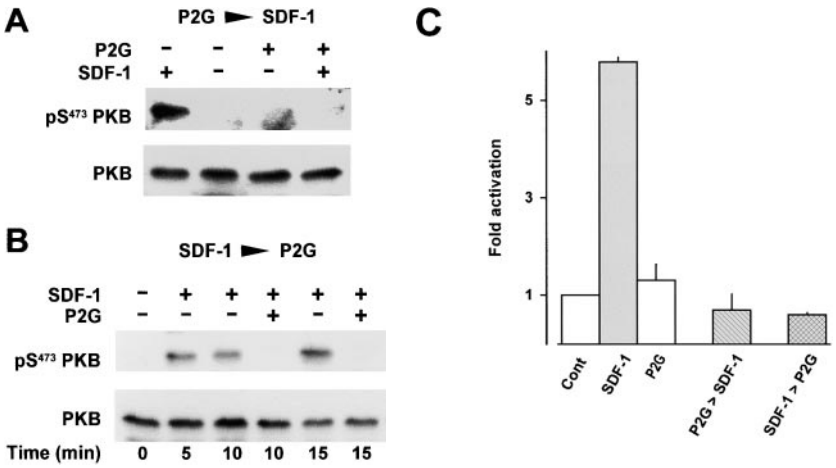

Figure 2. Effect of the CXCR4 antagonist, SDF-1P2G, on SDF-1–induced protein kinase B activation. (A) T cells were pretreated with 10 μM SDF-1P2G (P2G) or medium for 5 min and stimulated with 100 nM SDF-1 for 10 min. (B) The cells were stimulated with 100 nM SDF-1 for 5, 10, or 15 min. Where indicated, 10 μM SDF-1P2G (P2G) was added 5 min before termination of the stimulation. Activated ($pSer^{47}$ ^{3}PKB) and total protein kinase B(PKB) were determined as in the legend to Fig. 1 A. (C) Protein kinase B activity in immunoprecipitates. The cells were either treated with medium (Cont), 100 nM SDF-1 (SDF-1), or 10 μM SDF-1P2G (P2G) for 10 min, or treated with 10 μM SDF-1P2G for 5 min before stimulation with 100 nM SDF-1 for 10 min (P2G > SDF-1), or were stimulated with 100 nM SDF-1 and 5 min later treated with 10 μM SDF-1P2G for an additional 5 min (SDF-1 > P2G). Results are expressed as in Fig. 1 C. Similar results were obtained with cells from three different donors.

kinase B activity, whereas 10 μM SDF-1P2G had no effect. Pretreatment of the cells with SDF-1P2G fully inhibited the stimulation by SDF-1. Furthermore, addition of the antagonist after SDF-1 decreased protein kinase B activity to basal levels within 5 min. Two main conclusions can be drawn from these results: (a) continuous receptor–ligand interaction is required for sustained protein kinase B activation, and (b) CXCR4 must be expressed on the cell surface to remain accessible for SDF-1P2G.

Effect of the PI 3-Kinase Inhibitor Wortmannin on Protein Kinase B Activation. The specific PI 3-kinase inhibitor wortmannin (19) is known to block receptor-mediated activation of protein kinase B (18, 20, 21). Fig. 3 A shows that preincubation of IL-2–expanded T cells with 100 nM

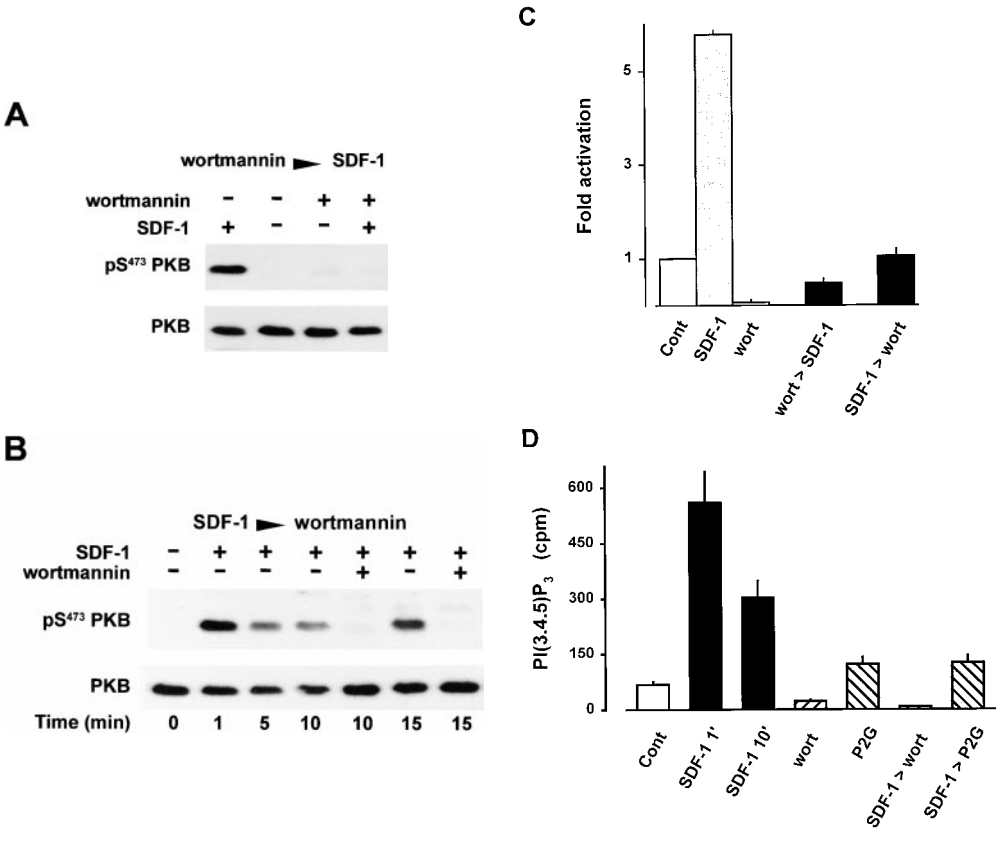

Figure 3. Effect of wortmannin on SDF-1–induced protein kinase B activation and PIP_3 production. (A) T cells were pretreated with 100 nM wortmannin for 10 min and stimulated with 100 nM SDF-1 for 10 min. (B) The cells were stimulated with 100 nM SDF-1 for 1, 5, 10, and 15 min. Where indicated, 100 nM wortmannin was added 5 min before termination of the stimulation. Activated ($pSer^{473}$PKB) and total protein kinase B (PKB) were determined as in the legend to Fig. 1 A. (C) Protein kinase B activity in immunoprecipitates. The cells were either treated with medium (Cont), 100 nM SDF-1 (SDF-1), or 100 nM wortmannin (wort) for 10 min, or were treated with 100 nM wortmannin for 10 min before stimulation with 100 nM SDF-1 for 10 min (wort > SDF-1), or were stimulated with 100 nM SDF-1 and 5 min later treated with 100 nM wortmannin for an additional 5 min (SDF-1 > wort). Results are expressed as in Fig. 1 C. D. PIP_3 formation in [^{32}P]orthophosphate-loaded T cells. The cells were either treated with medium (Cont), 100 nM SDF-1 (SDF-1 10′), 100 nM wortmannin (wort), 10 μM SDF-1P2G (P2G) for 10 min or with 100 nM SDF-1 for 1 min (SDF-1 1′), or were stimulated with 100 nM SDF-1 and 5 min later treated with 100 nM wortmannin (SDF-1 > wort) or 10 μM SDF-1P2G (SDF-1 > P2G) for an additional 5 min. Lipids were extracted, deacylated, and glycerophosphoinositides were separated by HPLC (duplicate determinations). Results are representative for at least three independent experiments.

wortmannin prevents SDF-1–mediated activation of protein kinase B. Furthermore, when wortmannin was added 5 or 10 min after stimulation of the cells with SDF-1, a rapid dephosphorylation of Ser^{473} of protein kinase B was observed (Fig. 3 B). Kinase activity measurements in immunoprecipitates prepared from wortmannin-treated cells confirm the findings (Fig. 3 C). Preincubation of serum-starved cells with 100 nM wortmannin abolished the basal protein kinase B activity and attenuated the stimulation by SDF-1. When wortmannin was added 10 min after treatment of the cells with SDF-1, protein kinase B activity was inhibited within 5 min, indicating that a continuous supply of 3-phosphoinositides is necessary to maintain the elevated protein kinase B activity. This conclusion was corroborated by measurements of the PIP_3 levels. As shown in Fig. 3 D, SDF-1 stimulated a rapid accumulation of PIP_3. A sevenfold increase of the basal level was obtained within 1 min that remained elevated three- to fourfold for at least 10 min. Treatment of the cells with 100 nM wortmannin decreased the basal content of PIP_3, whereas 10 μM SDF-1P2G had no effect. Both wortmannin and SDF-1P2G, when added during the course of SDF-1–dependent PIP_3 formation, rapidly reversed the activation of PI 3-kinase and the levels of PIP_3 returned to baseline values. These findings confirm that phosphorylation of protein kinase B is tightly coupled to the activation of PI 3-kinase and that sustained PIP_3 formation is critical for retaining protein kinase B in the active state. In addition, the results demonstrate that PI 3-kinase activation requires the continuous interaction of SDF-1 with CXCR4.

Effect of the SDF-1 Concentration on Protein Kinase B and ERK-2 Activation. The duration of protein kinase B activation was concentration dependent, lasting 5–10 min with 10 nM SDF-1 and up to 60–90 min with 1 μM SDF-1 (Fig. 4 A). However, consistent with a K_d of 3–4 nM for SDF-1 binding to CXCR4 (27), protein kinase B activity was stimulated 10-fold with 10 nM SDF-1 and maximum activation (15–16-fold) was obtained with 100 nM SDF-1 at 1 min after stimulus addition (Fig. 4 B). At 20 min, protein kinase B activity was less pronounced (threefold activation) but a similar concentration dependence was observed, suggesting that the receptor affinity does not change during the course of activation and that the duration of the agonist-induced response relies on the availability of SDF-1. Western blot analysis of the kinase content in each immunoprecipitate revealed equal amounts of enzyme, excluding the possibility that different recoveries account for the enhanced protein kinase B activities. Concentration-dependent prolonged stimulation was also found for ERK-2 (Fig. 4 C). At 1 μM SDF-1, activation was seen for up to 2 h.

CXCR4 Downregulation. Activation of G protein–coupled receptors generally results in rapid desensitization and downregulation. To test if the prolonged stimulatory capacity of CXCR4 is associated with reduced receptor internalization, surface expression of CXCR4 was measured by flow cytometry using PBMCs and the mAb 6H8. This antibody recognizes the NH_2 terminus of CXCR4 and shows

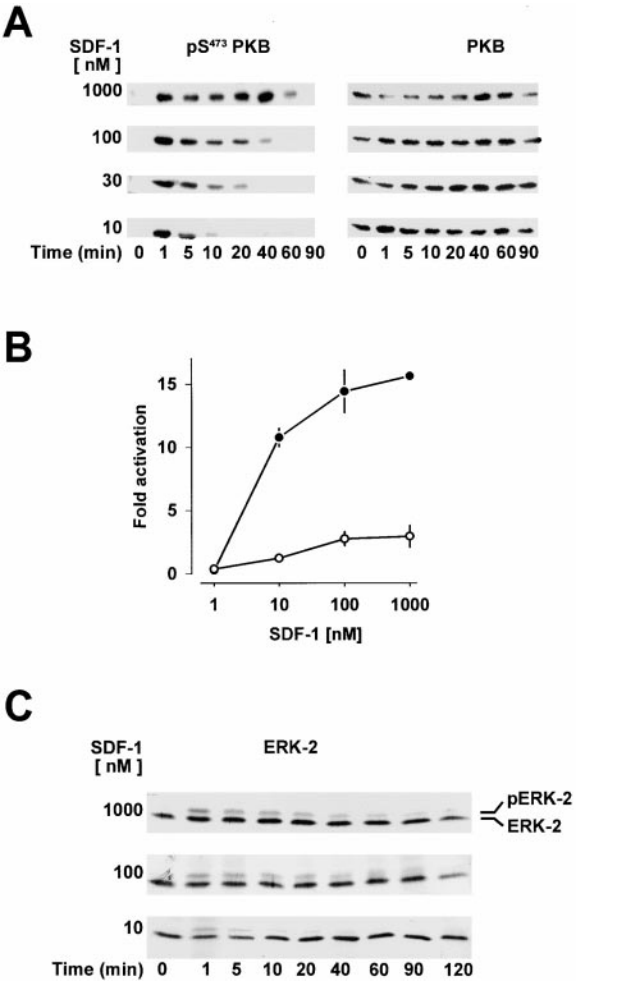

Figure 4. SDF-1 concentration dependence of prolonged protein kinase B and ERK-2 activation. (A) Western blot analysis of protein kinase B. T cells were stimulated with 10, 30, 100 nM or 1 μM SDF-1 for the indicated times. Activated ($pSer^{473}$ PKB) and total protein kinase B (PKB) were determined as in the legend to Fig. 1 A. (B) Protein kinase B activity in immunoprecipitates from lysates of cells stimulated with the indicated concentrations of SDF-1 for either 1 min (●) or 20 min (○). Results are expressed as fold activation. (C) Western blot analysis of ERK-2. T cells were stimulated with 10, 100 nM and 1 μM SDF-1 for the indicated times. Activated ERK-2 (pERK-2) corresponds to the band with retarded electrophoretic mobility. Results are representative for at least three independent determinations.

only borderline interference (<5%) with ligand binding and signal transduction (Thelen, M., and A. Amara, unpublished data). Upon stimulation with SDF-1, surface expression of CXCR4 is reduced in a concentration-dependent manner (Fig. 5). Receptor downregulation is biphasic, with rapid internalization during the first 5 min followed by a slower phase lasting for up to 10 h. Receptor endocytosis was already observed at 1 nM SDF-1 but was more pronounced with increasing agonist concentration. At 1 μM SDF-1, up to 80% of the CXCR4 receptors were internalized within 5 min compared with 20% at 1 nM SDF-1. Similar rates of internalization were found for other chemokine receptors, e.g., CCR5 on PBLs stimulated with RANTES, and for

CXCR4 in other cell types, such as T cell lines (Jurkat, CEM) and HeLa. The observed biphasic internalization kinetics are not unique to CXCR4 and cannot be fundamental for the prolonged signaling in response to SDF-1. The 20% of receptors that remain at the cell surface may therefore be sufficient to continuously trigger downstream signaling. This conclusion appears to contradict the signaling properties shown above: higher concentrations of SDF-1 cause more pronounced CXCR4 internalization and concomitantly induce more sustained protein kinase B activation (Fig. 4) that depends on continuous CXCR4 surface expression as shown with the receptor antagonist (Fig. 2). To test the possible explanation that rapid receptor recycling could account for the prolonged signaling capacity of CXCR4, we incubated T cells with SDF-1 for different times and after a brief acidic wash to remove bound and free ligand (11) measured receptor reexpression in the presence and absence of cycloheximide by flow cytometry. In agreement with previous reports (11), only ~20% of the initially surface-expressed receptors recycled within 20 min (data not shown), suggesting that receptor recycling may not be the predominant mechanism for the prolonged signaling by CXCR4, although not excluding it.

Restimulation of Protein Kinase B Activity with SDF-1. As shown in Figs. 1 and 4, stimulation of T cells with SDF-1 leads to an initial peak of protein kinase B activity followed by a protracted phase that depends on the concentration of SDF-1. This observation suggests that the duration of CXCR4 signaling may be limited by the availability of SDF-1 for receptor interaction. To test this possibility, the cells were stimulated with 100 nM SDF-1 for different times and protein kinase B activity was determined in immunoprecipitates. In parallel incubations, a second addition of 100 nM SDF-1 was made after 5, 20, or 60 min and protein kinase B activity was determined 1 min later, i.e., at 6, 21, or 61 min. Unexpectedly for a G protein–coupled receptor, up to 80% of the maximum protein kinase B activation could be recovered (Fig. 6 A). The effect was unique for SDF-1 stimulation of CXCR4. No enhancement of protein kinase B activity was obtained when other chemokine receptors, e.g., CCR2 (MCP-1), were restimulated, a finding that is in agreement with the general notion that chemokine receptors are rapidly desensitized.

When intracellular calcium mobilization was measured, CXCR4, as reported previously (32), was refractory to a second stimulation with SDF-1 (Fig. 6 B). Desensitization could be obtained in the presence of continuous recycling of receptors. A peak of intracellular free calcium concentration requires a burst inositol 1,4,5-triphosphate formation that triggers the sudden calcium release from intracellular

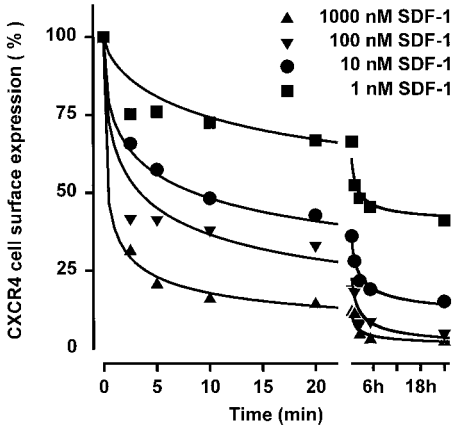

Figure 5. Downregulation of CXCR4 by SDF-1. PBMCs were incubated in RPMI supplemented with 10% FCS without (control, 100% expression) or with increasing concentrations of SDF-1 for the times indicated. Surface-expressed CXCR4 was detected with mAb 6H8 stained with PE-conjugated goat anti–mouse IgG using a FACScan™ flow cytometer. The percentage of CXCR4 surface expression was calculated from the relative fluorescence intensity (see Materials and Methods). The results shown are representative for four experiments obtained with cells from different donors.

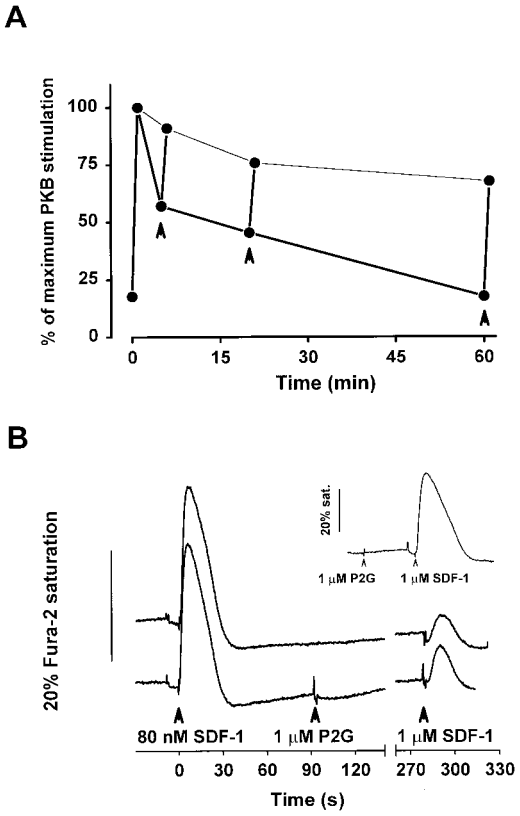

Figure 6. Restimulation of CXCR4 with SDF-1. (A) Protein kinase B activation. T cells were stimulated with 100 nM SDF-1 for the indicated times (points connected by solid line). In parallel samples, a second addition of 100 nM SDF-1 was made at 5, 20, or 60 min of stimulation (arrowheads), and the incubation continued for 1 min (points connected by thin line). The cells were lysed, and protein kinase B activity was measured in immunoprecipitates. Maximum protein kinase B activity obtained at 1 min of stimulation was set as 100%. (B) Changes in intracellular free calcium. Fluorescence of Fura-2–loaded T cells was recorded for 6 min in parallel samples. At time 0 the cells were stimulated with 80 nM SDF-1. Where indicated, 1 μM SDF-1P2G (P2G) was added 90 s later to one sample (bottom trace), and at ~280 s a second addition of 1 μM SDF-1 (SDF-1) was made to both samples. (Inset) 1 μM SDF-1P2G (P2G) does not desensitize the response to 1 μM SDF-1 (SDF-1). Results are representative for at least three independent experiments performed in duplicate with cells from different donors.

stores. Receptors that recycle to the cell surface would stimulate a continuous IP_3 formation, leading to moderate cytosolic calcium fluxes that are counterbalanced by Ca-ATPases. Thus, only marginal changes in intracellular free calcium would be observed. In the experiment shown in Fig. 6 B, parallel samples of Fura-2–loaded cells were stimulated with 80 nM SDF-1, which induced a rapid rise in intracellular free calcium. To one sample, a large excess of SDF-1P2G (1 μM) was added 90 s later to displace bound SDF-1 and to prevent binding of SDF-1 to potentially recycling receptors. The antagonist does not stimulate a calcium response per se (Fig. 6, inset). About 5 min after the first addition of SDF-1, both samples were restimulated with 1 μM SDF-1. Only a minimal response was observed in both cases, suggesting that in the presence of SDF-1P2G no receptors recycle to the cell surface that can be restimulated with SDF-1. Taken together, the results shown in Fig. 6 indicate that the CXCR4-mediated rise in cytosolic free calcium is desensitized with a single dose of agonist, whereas signaling leading to the activation of protein kinase B can be restimulated.

Role of SDF-1 Degradation. Recent reports show that the dipeptidyl dipeptidase IV CD26 cleaves several chemokines near the NH_2 terminus. Truncation of SDF-1 by CD26 abolishes functional responses such as chemotaxis and receptor internalization–dependent inhibition of HIV-1 infection (41). Because the availability of "active" (intact) SDF-1 could be limiting for CXCR4-dependent signaling, we tested the effect of the CD26 inhibitor, diprotin A, on protein kinase B activation. Fig. 7 illustrates that the activation obtained with 30 nM SDF-1 lasting for up to 20 min is prolonged up to 60 min in the presence of 4 mM diprotin A, indicating that inactivation of SDF-1 by CD26 significantly contributes to the termination of CXCR4-dependent signaling. Similar effects of diprotin A were observed when ERK-2 activation was measured. By contrast, addition of diprotin A did not cause a detectable prolongation of protein kinase B or ERK-2 activation when MCP-1 or MIP-1β was used as stimulus.

Discussion

Chemokines can mediate diverse cellular processes such as inflammatory responses, regulation of homing of lymphocytes, and embryogenesis. It is remarkable that chemokines govern these responses by binding to related seven transmembrane domain receptors coupled to G_i proteins. In this study, we investigated chemokine receptor–dependent signal transduction in human T cells to gain more information on the initiation of the functional responses elicited by different chemokines. The results show that CXCR4 is distinct from other chemokine receptors by its capacity to stimulate sustained activation of ERK-2, PI 3-kinase, and protein kinase B, kinases that have been implicated in cell proliferation, differentiation, and survival (42, 43). The SDF-1–CXCR4 couple is involved in homeostasis rather than inflammation, a notion that is supported by gene knockout experiments which revealed that both SDF-1 and CXCR4 are required for embryogenesis (7–9). Thus, it is conceivable that CXCR4 induces signal transduction that leads to proliferation and differentiation. Several lines of evidence suggest that PI 3-kinase–mediated activation of protein kinase B inhibits apoptosis and promotes cell survival (22–24, 26, 44). Accordingly, sustained activation of protein kinase B in the presence of SDF-1 could protect $CXCR4^+$ cells from undergoing apoptosis, a process that is critical for the activation of T cells (45, 46).

The biochemical mechanisms responsible for the sustained signaling by CXCR4 are not yet resolved. Gene disruption studies demonstrate that SDF-1 exclusively acts on CXCR4 (7–9), and the finding that *B. pertussis* toxin blocks the sustained signaling implies that initial coupling to a G_i protein is essential. The unusual signaling characteristics of CXCR4 are not restricted to specific cell types, as similar responses were obtained with several hematopoietic cells and with epithelium-derived HeLa cells. Our results demonstrate that the prolonged signaling depends on continuous receptor–agonist interaction at the cell surface, in agreement with a model of reversible ligand–receptor interaction as proposed for most G protein–coupled receptors. The observation that displacement of SDF-1 by a receptor antagonist causes immediate termination of cellular responses rules out the possibility that sustained signaling is mediated by a "second wave of signaling" stimulated by endocytosed or by sequestered and desensitized receptors (47). However, it can be envisaged that an association with adapter molecules preserves the receptor's signaling capacity and prevents its complete desensitization (47). This second phase of signaling may not depend on coupling of the receptor to heterotrimeric G_i proteins.

Rapid desensitization is a common hallmark of G protein–coupled receptors, leading to transient responses of short duration. In fact, most chemokines that we tested on T cells follow this scheme and induce only transient responses. Although RANTES at high concentrations can stimulate a sustained calcium influx that depends on tyrosine phosphorylation (40), protein kinase B and ERK-2 activation is transient and rapidly desensitized (not shown). These findings suggest that RANTES-mediated T cell activation is distinct from that induced by CXCR4. Desensitization is caused by receptor phosphorylation and is followed by rapid receptor internalization. Changes in the expression of selective chemokine receptors is a potential

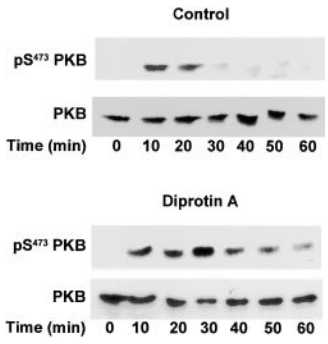

Figure 7. Inhibition of CD26/DPPIV (dipeptidyl dipeptidase IV) prolongs SDF-1 stimulated protein kinase B activation. T cells were incubated with 30 nM SDF-1 in the absence (Control) or presence of 4 mM of the CD26/DPPIV inhibitor diprotin A for the indicated times. Activated ($pSer^{473}PKB$) and total protein kinase B (PKB) were determined by Western blot analysis as in the legend to Fig. 1 A.

mechanism by which the responsiveness of hematopoietic cells is spatially and temporally coordinated (1, 5, 33). We and others found no significant difference between SDF-1–stimulated CXCR4 internalization and the internalization rate of other chemokine receptors (11, 48, 49). Rapid receptor recycling can lead to the continuous surface expression of active receptors and could promote sustained signaling. Reports by Amara et al. (11) and our own observations indicate that only a small fraction of internalized receptors (10–20%) recycle to the surface. However, this process is relatively slow (20–40 min) and is not unique for CXCR4. Calcium mobilization experiments as shown in Fig. 6 also do not provide evidence for extensive receptor trafficking. Thus, receptor recycling appears unlikely as the principal mechanism for sustained signaling but cannot be completely ruled out.

Clearance of inducible chemokines is necessary to balance immune responses. In the case of the constitutively expressed homing chemokines, elimination is a mandatory mechanism to regulate their functions. Deregulated production or degradation can have detrimental effects. CD26 participates in the process of T cell activation and when expressed on the surface of activated T cells, causes the inactivation of several chemokines (41). In the present study, we provide evidence that inhibition of CD26 results in prolonged SDF-1–induced signaling, suggesting that the dipeptidyl peptidase inactivates SDF-1. Therefore, at sites where it is highly expressed CD26 can attenuate CXCR4-mediated responses, such as lymphopoiesis. On the other hand, it has recently been reported that SDF-1 can bind with high affinity to cell surface proteoglycans without inducing cellular responses or interfering with SDF-1–induced signaling through CXCR4 (50). This interaction may lead to elevated local concentrations of active SDF-1. Here, we show that high concentrations of SDF-1 or multiple additions of the chemokine induce sustained responses in T cells (Figs. 4 and 6). However, the prolonged stimulation of CXCR4 leads to complete downregulation of the receptor (Fig. 5), suggesting that long-term exposure to SDF-1 limits the responsiveness of CXCR4. Taken together, CD26 and slow receptor downregulation rather than rapid desensitization determine the efficiency of SDF-1–induced signaling.

Stimulation of PIP_3 formation by G protein–coupled receptors is biphasic and involves two types of PI 3-kinases, PI 3-kinase$_\gamma$, which is activated by $\beta\gamma$ subunits of heterotrimeric G proteins (51), and a p85/p110 isoform, which is activated by tyrosine kinase–regulated pathways (52). It was suggested that the pronounced initial peak of PIP_3 formation depends on PI 3-kinase$_\gamma$ (53), whereas a more protracted PIP_3 formation is mediated by a p85/p110 isoform. Because activation of protein kinase B reflects the kinetics of PI 3-kinase stimulation, it is conceivable that the initial prominent peak of protein kinase B activation is mediated by the $\beta\gamma$ subunit–regulated PI 3-kinase$_\gamma$, whereas the more protracted activation is mediated by a p85/110 isoform (Fig. 1 B). Activation of the p85/110 isoform may be mediated by binding of an adapter to the receptor and could be independent of heterotrimeric G proteins (47). Alternatively, the early peak of protein kinase B activity could simply result from the simultaneous activation of a large number of surface-expressed receptors that become rapidly downregulated (Fig. 6).

Sustained activation has been shown to induce nuclear translocation of both ERK-2 and protein kinase B (54–57). In line with these reports, our preliminary experiments indicate that stimulation with SDF-1 can lead to nuclear translocation of both kinases (Ho, L., and M. Thelen, manuscript in preparation). Whereas the role of ERK-2 as activator of transcription is accepted (42), the nuclear translocation and possible gene activation by protein kinase B are not well understood. An involvement of protein kinase B in mRNA expression has been reported (58, 59), but the mechanism remains elusive. In two recent reports, it was shown that protein kinase B phosphorylates a nuclear forkhead transcription factor that subsequently accumulates in the cytosol (25, 26). Phosphorylation of this transcription factor causes its inactivation and the downregulation of apoptosis-mediating genes such as Fas ligand (25). Thus, although the role of protein kinase B in promoting cell survival is well documented, a general role of the kinase in gene activation remains to be established.

We observed that ELC also stimulates a moderately prolonged response via CCR7. Like SDF-1 and CXCR4 that are required for the engraftment of $CD34^+$ cells (60) and lymphopoiesis (9), ELC and CCR7 have been shown to be important regulators of T cell homing and maturation (61–63). It is plausible that chemokine receptors that mediate hematopoiesis perform this function via induction of sustained signals. Maturation of T cells may require the repetitive interaction with antigen-presenting cells (64). In this respect, a stable source of SDF-1 could hold $CXCR4^+$ cells in place and concomitantly induce sustained activation of mitogenic and survival pathways. The gradual loss of responsiveness to SDF-1 over 24 h would ensure that matured cells dissociate from antigen-presenting cells. Thus, it is conceivable that CXCR4 is capable of providing costimulatory signals in lymphocyte maturation.

We thank Drs. Fernando Arenzana-Seisdedos and James Hoxie for supplying the antibodies and Dr. Beatrice Dewald (Theodor Kocher Institute, University of Bern, Switzerland) for critical reading of the manuscript. Donor blood buffy coats were provided by the Swiss Central Laboratory Blood Transfusion Service.

This work was supported by the Swiss National Science Foundation (to M. Thelen).

Submitted: 14 February 2000
Revised: 4 May 2000
Accepted: 16 May 2000

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