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Optimal Chemotactic Responses of Leukemic T Cells to Stromal Cell-Derived Factor-1 Requires the Activation of Both Class IA and IB Phosphoinositide 3-Kinases

Adam P. Curnock, Yannis Sotsios, Karen L. Wright, and Stephen G. Ward

Stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 are a multifunctional chemokine/receptor system with essential roles in the development of the immune system and other aspects of embryogenesis, including vascularization and organ development. SDF-1 is also a potent chemotactant for T cells and has roles in both inflammation and immune homeostasis. Our group has previously demonstrated that phosphoinositide 3-kinase (PI 3-kinase) is activated in SDF-1-stimulated T cells and is indeed required for SDF-1-mediated chemotaxis. In this study Jurkat clones were established, stably expressing dominant negative constructs of class IA and class IB PI 3-kinases under the control of the tetracycline off inducible gene system, to determine the relative roles of these PI 3-kinases in SDF-1 signaling. Our results show that expression of either kinase-dead PI3Kγ (KD-PI3Kγ) or Δp85 (a construct unable to bind class IA p110α, -β, or -δ) leads to a partial inhibition of SDF-1-stimulated protein kinase B phosphorylation, but had no effect on SDF-1-induced phosphorylation of the mitogen-activated protein kinase ERK1/2. Functional studies demonstrated that expression of KD-PI3Kγ markedly inhibited SDF-1-mediated chemotaxis, typically eliciting 40–60% inhibition. Interestingly, the expression of Δp85 also leads to inhibition of the SDF-1-mediated chemotactic response, albeit to a much lesser extent than achieved with the KD-PI3Kγ mutant, typically in the range of 20–40% inhibition. Furthermore, the inhibition of chemotaxis by the expression of dominant negative class IA or class IB PI 3-kinases could be enhanced by the presence of the PI 3-kinase inhibitor LY294002. Together, these results demonstrate that optimal chemotactic response of leukemic T cells to SDF-1 requires the activation of both class IA and class IB PI 3-kinases. The Journal of Immunology, 2003, 170: 4021–4030.

The human chemokine system is comprised of ~50 chemokines and 20 G protein-coupled, seven-transmembrane chemokine receptors and is divided into four groups, C, CC, CXC, and CX3C chemokines, based upon the positioning of N-terminal cysteine residues (1). Chemokines play a central role in the development and regulation of the immune system. Primarily, they are responsible for the directional migration, or chemotaxis, of leukocyte populations, where they coordinate the homing of lymphocyte populations to specific lymphoid tissues and the recruitment of leukocytes to sites of infection or tissue damage. In addition to their chemotactic function, chemokines are implicated in other biological events, including cell growth, lymphopoiesis, vascularization, organ development, HIV pathogenesis, and tumor metastases (1–8). The realization of the importance of chemokines in immune function has encouraged an escalation of research into their roles in regulating the key processes, actin polymerization and cell polarization, underlying chemokine-mediated chemotaxis (3, 4, 9–15).

PI 3-kinases catalyze the phosphorylation of phosphatidylinositol lipids (PtdIns) at the 3’ position of the inositol ring to produce phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) (16). The levels of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are acutely regulated by class I PI 3-kinases in response to receptor stimulation and function as second messengers by recruiting pleckstrin homology (PH) domain-containing cytosolic proteins to the membrane, enabling them to perform either adaptor or catalytic roles (16–19). Class IA PI 3-kinases are heterodimeric proteins, comprising a 110-kDa catalytic subunit and a prototypical regulatory subunit of 85 kDa that is responsible for regulating the catalytic domain to the membrane by interactions with tyrosine-phosphorylated proteins via its SH2 domains. Three mammalian forms of the catalytic subunit (p110α, p110β, and p110δ) and five different regulatory subunits (p85α, p85β, p55γ, and two splice variants of p85αp50α and p55α) have been identified (20–23). A G protein-coupled PI 3-kinase activity, consisting of a 120-kDa catalytic subunit, named p110γ, and its 101-kDa regulatory subunit, have been purified and cloned (24, 25). The N-terminal region of p110γ diverges considerably from class IA PI 3-kinases, does not interact with p85 regulatory proteins, and has been designated a separate subclass, class IB (26).

Studies using green fluorescent protein-tagged PH domains that bind selectively to PtdIns(3,4,5)P3 or PtdIns(3,4,5)P3-specific Abs...
have revealed that PtdIns(3,4,5)P_2 accumulates rapidly at the leading edge of chemottractant-stimulated cells (11, 12). Furthermore, it has been demonstrated that protein kinase B (PKB), a major effector of the PI 3-kinase-dependent signaling cascade that interacts with PtdIns(3,4,5)P_2 and PtdIns(3,4)P_2 via its PH domain, colocalizes with these lipids and filamentous actin at the leading edge (13). These studies suggest that the polarized activation of PI 3-kinase and subsequent PtdIns(3,4,5)P_2-dependent recruitment of PKB and possibly other PH domain-containing proteins are crucial early events in the detection of a chemottractant gradient. Very recent studies have implicated intimate roles for PI 3-kinase, the lipid products of PI 3-kinase and Rho family GTPases in the generation of a positive feedback loop that produces an internal PtdIns(3,4,5)P_2 gradient, exceeding that of the external chemottractant gradient (14, 15). This provides a mechanism for the detection of very shallow chemotactrant gradients, enabling polarization and directional movement of cells. Recent exciting studies have also revealed a role for phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in maintaining cell polarity by localizing at the rear and sides of cells, thus enhancing the polarized accumulation of PtdIns(3,4,5)P_2 at the leading edge (27, 28).

Despite these exciting developments, it is unclear which PI 3-kinase isoforms are involved in the accumulation of PtdIns(3,4,5)P_2 at the leading edge (27, 28). Analogous to Src family kinases, which can function as both growth-promoting and growth-inhibiting factors, PI 3-kinases could be involved in regulating cell growth by controlling the activity of a variety of downstream signaling molecules.

Using a pharmacological approach, it is not possible to assign the chemokine-stimulated accumulation of PtdIns(3,4,5)P_2 and the chemotactic response to a particular PI 3-kinase isoform, since available inhibitors do not exhibit sufficient isoform specificity. Genetic approaches have revealed a key role for p110α in chemotactic responses (35–37). However, studies with mice deficient in class IA PI3K activity are limited by impaired viability and the fact that p85α−/− and p85β−/− mice retain the expression of at least one other isoform of the adaptor subunit (38–40). A common molecular approach used to assess the role of an enzyme in cellular responses is to establish stable transfectants that overexpress dominant negative forms of class IA PI3K constructs in the correct orientation. The identity and orientation of the PI 3-kinase inserts were confirmed by sequencing using forward and reverse primers flanking the insertion site on pUHD10-3 hygro. The forward primer sequence was CCAATTGAAAGCAGCGGA, and the reverse primer sequence was GACTTCTAGTTGGTTTGTG. To confirm the identity of KD-PI3K inserts, primers flanking the catalytic domain region were used: the forward primer sequence was CAGAAATTTGAATCTCCCCCA, and the reverse primer sequence was TGATAGACCGCTAGGATCG. A single guanosine to adenosine substitution in codon 799, resulting in a lysine to arginine substitution, confirmed the identity of the kinase-dead PI3Kγ construct.

### Cell culture

The human leukemic T cell line Jurkat, stably expressing the pUHD15-1-neo plasmid (tetracycline-off Tet-Off Jurkat cells), was purchased from Clontech (Basingstoke, U.K.). Cells were cultured in humidified incubators in 5% CO_2 at 37°C in RPMI 1640 medium, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For selection of Tet-Off clones expressing both regulatory and response vectors, the medium was supplemented with G418 (500 μg/ml), hygromycin B (300 μg/ml), and 2 μg/ml tetracycline.

### Dominant negative PI 3-kinase cloning strategy

Dominant negative constructs of class IA and IB PI 3-kinases were subcloned into the response vector pUHD10-3 hygro, which contained a hygromycin resistance gene, for direct selection of transfectants. For class IA PI3K, a bovine p85α regulatory subunit construct with a deletion of the inter-SH2 domain (aa 479–513, which constitute the p10 binding domain) was used. This Δp85 is unable to bind to p10 catalytic domains, but retains its adapter function via its two SH2 domains, SH3, and proline-rich regions, and so overexpression of Δp85 prevents the recruitment and activation of the catalytic subunit by out-competing endogenous p85 for binding sites. For the class IA PI3 kinase, KD-PI3Kγ was used. KD-PI3Kγ has a single-point mutation in its catalytic domain, K799R, rendering it inactive. Both constructs were tagged by the decapeptide recognized by the c-Myc mAb 9E10 to enable screening for expressing clones (43). Myc-tagged Δp85 was excised from the pBlueScript-N-Myc vector using SacI and EcoRI, and Myc-tagged WT and KD-PI3Kγ were excised from the pCDNA3 using KpnI and XbaI. All inserts were blunt ended on both the 3′ and 5′ ends using T4 polymerase, followed by the Klenow fragment of DNA polymerase. The response plasmid pUHD10-3 hygro was linearized with XbaI, followed by blunt ending and removal of 3′-OH groups by treatment with calf intestinal phosphatase to minimize rescaling of empty pUHD10-3 hygro. Ligation reactions of linearized, blunt-ended pUHD10-3 hygro with each of the blunt-ended Myc-tagged PI 3-kinase constructs were conducted and ligation products were used to transform Escherichia coli strain DH5α. Analytical restriction digests were performed to identify transformants with pUHD10-3 hygro-containing inserts in the correct orientation. The identity and orientation of the PI 3-kinase inserts were confirmed by sequencing using forward and reverse primers flanking the insertion site on pUHD10-3 hygro. The forward primer sequence was CTCMTAGAAAGCAGCGGA, and the reverse primer sequence was GCATTCTAGTTGGTTTGTG. To confirm the identity of KD and WT p110α inserts, primers flanking the catalytic domain region were used: the forward primer sequence was CAGAAATTTGAATCTCCCCCA, and the reverse primer sequence was TGATAGACCGCTAGGATCG. A single guanosine to adenosine substitution in codon 799, resulting in a lysine to arginine substitution, confirmed the identity of the kinase-dead PI3Kγ construct.

### Transfection and selection of Tet-Off dominant negative PI 3-kinase Jurkat clones

The pUHD10-3 dominant-negative PI3K constructs were linearized with FspI and purified by gel electrophoresis, chloroform extraction, and ethanol precipitation. Jurkat cells already stably expressing the pUHD15-1-neo plasmid (Tet-Off Jurkats) were transfected with 10 μg of purified DNA by

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**Materials and Methods**

**Reagents**

A bovine p85α regulatory subunit construct with a deletion of the inter-SH2 domain (aa 479–513, which constitute the p10 binding domain) was a gift from Dr. M. Kasuga (Kobe University, Kobe, Japan).

**Type (WT) and KD-PI3K constructs (K799R)** were gifts from Dr. H. Takeda (Kobe University School of Medicine, Kobe, Japan). The response vector pUHD10-3 hygro was provided by M. J. Welham (University of Bath, Bath, U.K.). SDF-1α and human insulin-like growth factor I (IGF-I) was purchased from R&D Systems (Abingdon, U.K.). A c-Myc mAb 9E10 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific polyclonal Abs, recognizing PKB phosphorysine 473 and ERK1/2 (p42/p44) phosphorylated at threonine 202 and tyrosine 204, were purchased from New England Biolabs (Knovel Pierce, U.K.). G418, hygromycin B, pertussis toxin, LY294002, and wortmannin were purchased from Calbiochem (Nottingham, U.K.). Histone 2B (H2B) was supplied by Roche (Lewes, U.K.). The ECL system and [γ-32P]ATP were obtained from Amersham International (Little Chalfont, U.K.). Cell culture reagents were purchased from Life Technologies (Paisley, U.K.). All other reagents were purchased from Sigma-Aldrich (Poole, U.K.). Where appropriate, stock solutions of drugs were prepared in a suitable solvent, aliquoted, and stored at −20°C. Final dilutions were made immediately before use.
and after the indicated treatment regimens the reactions were terminated by adding SDS-PAGE sample buffer and boiling for 5 min.

**Flow cytometry**

Cells (1 × 10^6) were washed twice in PBS and resuspended in 100 μl of anti-CXCR4 (12G5, 10 μg/ml) or corresponding IgG2a isotype control, diluted in PBS/10% FBS, and incubated for 45 min on ice. After washing, cells were incubated in 100 μl of anti-mouse IgG-FITC secondary Ab for 1 h. After a further round of extensive washing in TBS/Tween, membranes were incubated with an appropriate HRP-conjugated secondary antibody.

**Cell lysis**

Cells (1 × 10^6/sample) were incubated in 1.5-ml microfuge tubes at 37°C, and after the indicated treatment regimens the reactions were terminated by pelleting the cells in a microfuge for 10 s, aspiration of the supernatant, and then resuspending in 500 μl of ice-cold lysis buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM MgCl_2, 1 mM CaCl_2, 10% glycerol (w/v), 1% Nonidet P-40 (w/v), and protease inhibitors, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 mM sodium orthovanadate).

Lysates were incubated on a rotator for 20 min, and nonsoluble material was removed by spinning in a microfuge at 14,000 rpm for 10 min. The protein levels in supernatants were determined by the bicinchoninic acid protein assay (Pierce and Warriner, Chester, U.K.) and adjusted to equal protein concentration by dilution with lysis buffer where necessary.

**PKB assays**

PKB assays were conducted following the procedure described by Reif et al. (44, 45). Cell lysates were preabsorbed with protein A-Sepharose (50 μl of a 50/50 slurry) for 1 h on a rotator and spun at 15,000 rpm to pellet the Sepharose beads, and the lysates were transferred to clean tubes. Anti-PKB Ab was preconjugated to protein G-agarose beads for 1 h at 4°C on a rotator (at 2 μg of Ab/20 μl of protein G-agarose (50/50 slurry)). Before use, anti-PKB-protein G-agarose beads, were washed three times in lysis buffer, and 20 μl was added to each sample, followed by incubation for 1–2 h on a rotator at 4°C. Immunoprecipitates were washed twice in lysis buffer twice in high salt wash buffer (100 mM Tris (pH 7.5), 500 mM LiCl, and 1 mM EDTA), and once in PKB assay buffer (50 mM Tris (pH 7.5), 10 mM MgCl_2, and 1 mM DTT). The last wash was removed as completely as possible, and reactions were started by adding 15 μl of PKB reaction buffer and 20 μl of [γ-32P]ATP (2.5 μM of H2B, 500 nM protein kinase inhibitor, and 3 μCi of [γ-32P]ATP). The reaction was allowed to proceed for 30 min at 25°C with constant agitation and was terminated by adding SDS-PAGE sample buffer and boiling for 5 min. Samples were resolved on 14% SDS-PAGE gels. The lower half of the gel was dried and analyzed by autoradiography, followed by densitometric analysis (Gene Tools; Syngene, Cambridge, U.K.) to determine 32P incorporation into H2B.

**Immunoblotting**

Cell lysates were boiled for 5 min in sample buffer, and samples were loaded, at volumes corresponding to equivalent amounts of protein, onto 7.5% SDS-PAGE gels. After electrophoresis, resolved proteins were transferred onto nitrocellulose membranes and probed with the appropriate Abs. Briefly, membranes were blocked in TBS (10 mM Tris (pH 7.5) and 100 mM NaCl)/5% nonfat milk for 2 h, followed by overnight incubation with primary Ab (at 0.5–1 μg/ml diluted in TBS/0.1% Tween/0.01% sodium azide) at 4°C on a platform shaker. After extensive washing in TBS/Tween, membranes were incubated with an appropriate HRP-conjugated secondary Ab for 1 h. After a further round of extensive washing in TBS/Tween, membranes were developed using the ECL system. When necessary, membranes were stripped for reprobing by incubation in stripping buffer (62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM 2-ME) at 60°C for 30 min.

**Results**

**Expression of dominant negative class IA and IB PI 3-kinases is tightly regulated using the tetracycline off (Tet-Off) inducible gene system.**

Tet-Off Jurkat clones, expressing dominant-negative class IA (Δp85, a p85 deletion mutant lacking aa 479–513 of the inter-H2 domain, which constitutes the p110 binding domain and hence is unable to bind p110α, β, or δ catalytic subunits) and class IB (KD-Pt3Ky) PI 3-kinases were selected that exhibited tight regulation of expression, with minimal leakiness in the presence of tetracycline (Fig. 1A). High and intermediate expressing clones for Δp85 (clones 1 and 2) and KD-Pt3Ky clones (clones 3 and 4, respectively) are demonstrated in Fig. 1A. The Tet-Off system allowed the levels of expression of Δp85 and KD-Pt3Ky to be regulated dose-dependently over a range of tetracycline concentrations (IC_{50} = 0.3 ng/ml; Fig. 1B). FACs analysis of expressing clones revealed levels of CXCR4 surface expression similar to those seen in empty vector control cells (Fig. 1C).

**SDF-1-stimulated PKB activation is inhibited by the expression of class IA and IB dominant-negative PI 3-kinases.**

The serine/threonine kinase, PKB, is a major downstream effector of PI 3-kinase activity, whose recruitment and subsequent activation are entirely dependent on the lipid products of PI 3-kinase (46). Several studies have reported the activation of PKB following SDF-1 stimulation of leukocytes (47–49), and a specific role for PKB in the polarization of neutrophils toward a chemotactic gradient has been determined (12, 13). Therefore, we verified whether the PI 3-kinase mutants were indeed acting to disrupt PI 3-kinase activation by assessing their effect on PKB activation in the high expressing KD-Pt3Ky (clone 3) and Δp85 (clone 1) clones using two approaches. Firstly, immunoblotting for phospho-Ser^{473} (a residue that is phosphorylated during the activation of PKB) and, secondly, using a specific PKB-specific antibody to detect PKB phosphorylation and activity (Fig. 2). SDF-1 (0.3 ng/ml) stimulation of the T-cell line resulted in the phosphorylation of PKB at the Ser^{473} residue, but in the presence of Δp85 or KD-Pt3Ky, PKB phosphorylation and activity were significantly reduced (Fig. 2, A and B). This inhibition of PKB phosphorylation and activity by KD-Pt3Ky and Δp85 corroborates the results of previous studies that show that these constructs do indeed disrupt PKB phosphorylation and activity by both KD-Pt3Ky and Δp85.
PI3K/PKB activation, probably by disrupting their respective endogenous PI 3-kinase activities.

Class I dominant negative PI 3-kinases do not inhibit SDF-1-stimulated ERK 1/2 activation

Several studies have demonstrated activation of the mitogen-activated protein kinase (MAPK) ERK1/2 in response to SDF-1 stimulation (47, 49, 52), but the role of ERK1/2 in chemokine mediated-chemotaxis is contentious. While the MAPK kinase inhibitor PD98059 was shown to partially inhibit SDF-1, eotaxin, and macrophage inflammatory protein-3α-induced chemotaxis in T cells and eosinophils, respectively (47, 53, 54), the chemotactic response of neutrophils to IL-8 was unaffected by PD98059 (55). This may reflect functional diversity, where chemokines couple to distinct signaling pathways to evoke a particular response.

The effects of dominant negative, class I PI 3-kinases on SDF-1-mediated ERK 1/2 activation were assessed using a phosphospecific Ab recognizing phospho-ERK1/2. An SDF-1 time-course experiment in nonexpressing, dominant negative PI 3-kinase clones demonstrated a rapid and transient activation of ERK1/2, peaking at 2 min and declining after 5 min (Fig. 3, top panels). The expression of KD-PI3Kγ or Δp85 did not alter the profile of SDF-1-stimulated ERK1/2 phosphorylation in terms of either the magnitude or kinetics of phosphorylation.

Expression of dominant negative class IA and class IB PI 3-kinases inhibits SDF-1-mediated chemotaxis

We and others have previously shown that SDF-1 stimulates a robust, wortmannin-sensitive chemotactic response in Jurkat T cells, suggesting that it is a PI 3-kinase-dependent event (33, 34, 47). The use of Jurkat clones, inducibly expressing class IA and class IB dominant negative PI 3-kinases, under the control of the Tet-Off system enabled the roles of individual PI 3-kinase isoforms in SDF-1-mediated chemotaxis to be assessed. In a noninduced...
state, dominant negative PI 3-kinase clones exhibited a characteristic bell-shaped chemotactic response to a range of SDF-1 concentrations (Fig. 4). Although individual clones exhibited different levels of chemotaxis, in terms of the number of cells migrating the overall profiles of chemotaxis were similar, with maximal chemotaxis observed in response to 1 nM SDF-1 in all clones. In the absence of a chemotactic gradient (equal concentrations of SDF-1 in the upper and lower wells), the migration of cells was not affected by the expression of mutated PI 3-kinase constructs in any clone examined (data not shown).

Clones expressing class I dominant negative PI 3-kinases exhibited impeded cell migration toward an SDF-1 gradient over a range of SDF-1 concentrations (Fig. 4, A and B). Compared with the maximal chemotaxis attained in respective noninduced cells in response to 1 nM SDF-1, the expression of KD-PI3Kγ inhibited chemotaxis by 62.5% (clone 3) and 36.8% (clone 4). In contrast, the expression of Δp85 inhibited chemotaxis by 44.7% (clone 1) and 25.3% (clone 2). Thus, the degree of inhibition of chemotaxis generally correlates with the level of expression of the dominant negative PI 3-kinase (Table 1).

Expression of dominant negative class IA, but not class IB, PI 3-kinases inhibits IGF-I-mediated chemotaxis

The ability of the PI 3-kinase mutants to influence migratory responses to other chemotactic factors that use receptor classes such as the tyrosine kinase-linked receptor for IGF-I was also investigated. While the expression of KD-PI3Kγ had no effect on the chemotactic responses to IGF-I, these responses were abrogated by the expression of the Δp85 mutant (Fig. 5). This confirms that the inhibitory effects of the KD-PI3Kγ mutant on cell migration are context dependent, being able to influence responses initiated by chemotactic agents acting via G protein-coupled receptor, but not those acting via tyrosine kinase-linked receptors.

Δp85 and KD-PI3Kγ inhibition of SDF-1-mediated chemotaxis can be further abrogated by the PI 3-kinase inhibitor LY294002 and pertussis toxin

If, as indicated in the above data, both class IA and class IB PI 3-kinases are indeed required for an optimal chemotactic response to chemokine gradients, one might predict that the partial inhibition of chemotaxis observed in Δp85- and KD-PI3Kγ-expressing...
cells would be further inhibited in the presence of a pharmacological inhibitor of PI 3-kinase activity. To address this hypothesis, chemotaxis assays with 1 nM SDF-1 were conducted on KD-PI3Kγ-expressing clones preincubated with 30 μM LY294002 in either the absence or the presence of tetracycline. Incubation of cells with a range of LY294002 concentrations from 0.1–30 μM had no effect on the basal migration of cells alone (data not shown). When incubated with vehicle alone, the expression of KD-PI3Kγ inhibited SDF-1-mediated chemotaxis by ~60%. However, in the presence of LY294002, the chemotactic response in KD-PI3Kγ-expressing cells was reduced by ~80% (Fig. 6A). Similarly, addition of LY294002 to Δp85-expressing cells resulted in ~75% inhibition of SDF-1-mediated chemotaxis compared with the 20% inhibition observed upon expression of Δp85 in the absence of the inhibitor. The inhibitory effects of LY294002 on migration in the presence of either KD-PI3Kγ or Δp85 were concentration dependent (Fig. 6, B and C). This partial inhibition of SDF-1-induced chemotaxis by cells expressing individual class IA and IB dominant-negative PI 3-kinase isoforms and the further inhibition seen in the presence of a pharmacological inhibitor of PI 3-kinase suggest a role for multiple class I PI 3-kinases in the SDF-1-mediated chemotactic response in human T cells. As expected, pertussis toxin completely abrogated the migratory responses of nonexpressing KD-PI3Kγ and Δp85 clones to SDF-1 as previously reported (Fig. 7). Similarly, the addition of pertussis toxin abrogated the residual migratory activity that was observed upon the expression of either KD-PI3Kγ or Δp85, thus confirming that the biochemical signals supporting the mutant resistant migration were mediated via G protein-dependent routes.

**Discussion**

This study has focused on the exclusive SDF-1/CXCR4 chemokine/receptor pair to investigate the regulation of chemotaxis and chemokine signaling by class I PI 3-kinases in human T cells. The use of the Tet-Off inducible gene system (56) to regulate the expression of dominant negative PI 3-kinases has allowed the relative roles of individual class I PI 3-kinase isoforms in SDF-1-mediated chemotaxis and signaling to be accurately assessed. Specific inhibition of class IA or class IB PI 3-kinases by the
inducible expression of Δp85 and KD-PI3Kγ, respectively, resulted in a partial inhibition of SDF-1-mediated chemotaxis that could be further inhibited by the PI 3-kinase inhibitor, LY294002. Taken together, these results provide evidence for the requirement of both class IA and class IB PI 3-kinase isoforms in the coordination of optimal SDF-1-induced chemotaxis.

The idea that multiple isoforms of PI 3-kinase are required for chemokine signaling is beginning to receive credence as more studies demonstrate the involvement of different PI 3-kinase isoforms in chemokine-mediated responses (35, 47, 57). Earlier studies (33, 47) presenting apparently conflicting results, implicating either class IB or class IA PI 3-kinases downstream of SDF-1 merely reflect differences in the methods used to measure PI 3-kinase activity and, in fact, independently demonstrate the importance of both PI 3-kinase isoforms. Several lines of evidence strongly support the involvement of the Gβγ-dependent PI3Kγ in mediating PtdIns(3,4,5)P3 accumulation. Firstly, the importance of a functional PI3Kγ in chemokine-mediated chemotaxis has been demonstrated in studies of PI3Kγ−/− mice, where chemotaxis in response to a range of chemoattractants was partially inhibited in PI3Kγ−/− leukocytes (35–37). Secondly, neutrophils from PI3Kγ−/− mice are unable to produce PtdIns(3,4,5)P3 in response to the CXC chemokine IL-8 (35). Finally, the accumulation of PtdIns(3,4,5)P3 in cell lines stimulated by SDF-1 and MCP-1 can be completely inhibited by pretreatment with the G protein inhibitor pertussis toxin (47, 57–59). However, there is also compelling evidence for the involvement of class IA in chemokine signaling. For example, chemokine-mediated chemotaxis in leukocytes from PI3Kγ−/− was only partially abrogated (35), while chemoattractant-induced actin polymerization was not inhibited (36). Similarly, the pertussis toxin sensitivity of chemokine-stimulated PtdIns(3,4,5)P3 accumulation does not necessarily exclude the involvement of class IA PI 3-kinases. Indeed, the activation of class IA PI 3-kinases downstream of G protein-coupled receptors in a variety of leukocytes has been shown (60, 61), and class IA PI 3-kinases can be synergistically activated by Gβγ subunits and tyrosine phosphoproteins at least under in vitro conditions (30, 31). It has also been postulated that activation of class IB PI 3-kinases may influence the activation of class IA PI 3-kinases (62). In this respect we have previously shown that activation of PI3Kγ and that of class IA PI 3-kinases following chemokine stimulation exhibit distinct kinetics, with PI3Kγ activity peaking within 15–30 s, while p85/p110 PI 3-kinase activation occurs within minutes after exposure to chemokine (3). The most likely route for chemokine-mediated activation of class IA PI 3-kinases is via G protein-mediated activation of tyrosine kinase activity and the subsequent recruitment and activation of p85/p110 heterodimers via phosphotyrosine interactions. In fact, several chemokines stimulate tyrosine kinase activity (4, 59), and one study has demonstrated the direct activation of Src kinases by GTP-bound Gαi (63). Furthermore, in vitro assays indicate that class IA PI 3-kinases are activated by SDF-1 and RANTES in T cells (47, 57), and in vivo, the use of a dominant negative class IA construct, indicates that they are indeed necessary for chemokine-induced chemotaxis in T cells (33).

The data presented in this report demonstrate partial inhibition of SDF-1-mediated chemotaxis by the expression of dominant-negative forms of either class IA or IB PI 3-kinases in human T cells. The inhibition of residual chemotactic activity in both Δp85- and KD-PI3Kγ-expressing clones by a pharmacological inhibitor of PI 3-kinase further supports the role of multiple PI 3-kinase isoforms in chemokine-mediated responses. Alternatively, there is a small possibility that incomplete inhibition of migration by the individual mutants may simply reflect their inability to induce complete inhibition of the respective PI 3-kinases. This possibility cannot be entirely ruled out, and certainly the degree of inhibition of chemotaxis varies with the level of expression of mutants observed in different PI 3-kinase-expressing clones. In addition, there is evidence that different p85 regulatory subunits are able to associate with distinct proteins (64–66). Thus, Δp85 (a deletion mutant of p85α) may not be an effective competitor for some substrates recognized specifically by other class IA regulatory subunits and hence be unable to completely inhibit catalytic subunit recruitment.

Table I. Inhibition of SDF-1-mediated chemotaxis by dominant negative PI 3-kinases is dependent on their level of expression

<table>
<thead>
<tr>
<th>Tet-Off PI3K Clones</th>
<th>Δp85 Clone 1</th>
<th>Δp85 Clone 2</th>
<th>KD-PI3Kγ Clone 3</th>
<th>KD-PI3Kγ Clone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Inhibition of chemotaxis (n = 6)</td>
<td>44.7 ± 9.4</td>
<td>25.3 ± 6.6</td>
<td>62.5 ± 9.3</td>
<td>36.8 ± 3.2</td>
</tr>
<tr>
<td>p Value (t test)</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.001</td>
<td>≤0.01</td>
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a Chemotaxis experiments were carried out, as described in Materials and Methods, on Tet-Off Jurkat clones containing the indicated PI 3-kinase constructs. For each clone the chemotactic response to SDF-1 (1 nM) was compared between nonexpressing (plus tetracycline) and mutant PI 3-kinase-expressing (minus tetracycline) cells. The data are presented as inhibition of SDF-1-mediated chemotaxis resulting from the expression of PI 3-kinase constructs (mean ± SEM). The results represent the mean of at least four independent experiments, using four replicates per data point. Data were analyzed by ANOVA, followed by Student’s t test, to compare responses in the presence and absence of Tet at individual concentrations of SDF-1.

FIGURE 5. Effect of Δp85 and KD-PI3Kγ expression on IGF-1-stimulated chemotaxis. Tet-Off Jurkat clones (2 × 10⁶ cells/ml), stably transfected with KD-PI3Kγ clone 3 and Δp85 clone 2 as indicated, were incubated in the presence (●) or the absence (□) of 2 μg/ml tetracycline for 48 h, washed, and resuspended in RPMI/0.1% BSA. Cells (2 × 10⁶ cells/200 μl) were added to the upper wells of a chemotaxis chamber (NeuroProbe) above lower wells containing 1 ng/ml IGF-I as described in Materials and Methods. Chemotaxis across a 5-μm membrane was determined after a 2-h incubation at 37°C in 5% CO₂. Data are expressed as the mean chemotactic index (±SEM; n = 5), which is the ratio of cells migrating toward IGF-I vs cells randomly migrating. Data were analyzed by ANOVA, followed by Student’s t test to compare responses in the presence and the absence of Tet at individual concentrations of SDF-1 (*, p < 0.05).
The expression of class IA and IB dominant-negative PI 3-kinases inhibited SDF-1-stimulated activation of PKB, not only confirming the inhibition of their respective endogenous PI 3-kinase activities, but also suggesting a role for PKB downstream of chemokine signaling. In Dicyostelium, PKB membrane localization and activation are crucial steps in the detection of chemotactic gradients, and mutants deficient in PKB are unable to polarize and migrate very inefficiently toward the chemotactic agent (67). Evidence for a similar role for PKB in mammalian cells is beginning to emerge, where PKB is recruited to the leading edge, by interaction with PtdIns(3,4,5)P3 and PtdIns(3,4)P2 via its PH domain, and colocalizes with filamentous actin (12, 13).

Interestingly, the expression of dominant negative class I PI 3-kinases did not interfere with SDF-1-induced activation of ERK1/2. Previously, we and others have shown that chemokine-stimulated ERK1/2 activation in various leukocytes is inhibited by PI 3-kinase inhibitors, suggesting a PI 3-kinase-dependent activation of the MAPK pathway (47, 55, 68). However, in other studies chemokine-mediated activation of ERK1/2 has proven to be refractory to PI 3-kinase inhibition (69). These discrepancies may be explained in terms of different chemokines coupling to MAPK activation via distinct signaling pathways or the involvement of other PI 3-kinase isoforms. These discrepancies may be explained in terms of different chemokinesST2 coupling to MAPK activation via distinct signaling pathways or the involvement of other PI 3-kinase isoforms. For example, class II PI 3-kinase γ, which, incidentally, is insensitive to concentrations of wortmannin and activation. Another possibility is that overexpression of Δp85 may inhibit chemotaxis indirectly by its ability to sequester adapter proteins with its SH2, SH3, and proline-rich domains. However, this is unlikely as this study has demonstrated that overexpression of Δp85 specifically inhibits SDF-1-mediated PKB activation, but does not have any effect on SDF-1-induced ERK1/2 activation. It should be emphasized that even in the presence of a single dominant negative PI 3-kinase and optimal concentrations of PI 3-kinase inhibitor, cell migration was not completely abrogated. This suggests that other PI 3-kinase-independent pathways may contribute to the chemotactic response. Another possibility is the involvement of class II PI 3-kinases, which are less sensitive to PI 3-kinase inhibitors and are known to be activated by monocyte chemotactic protein-1 (57) and SDF-1 (unpublished observation).

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and LY294002 that are routinely used to inhibit class I PI 3-kinases (57, 70).

Studies using PI3K-y−/− knockout mice have shown that neutrophils exhibit differing degrees of inhibition of chemotaxis in response to different chemotactic agents (35). This study highlights the fact that class IA PI 3-kinases can make a significant contribution to the chemotactic response mediated by chemokines. However, since the Δp85 construct is able to interact with either p110α, -β, and -δ, these studies do not give us any insight into which catalytic domains are involved in SDF-1-mediated chemotaxis. The challenge now is to determine whether different chemokine receptors couple to distinct PI 3-kinase isoforms and, furthermore, whether the context of stimulation, i.e., specific ligands and/or cell type, dictates which PI 3-kinase isoform(s) is used. With regard to this, there is evidence that different p110 isoforms regulate distinct cellular processes in response to a single agonist, while different agonists can couple to the same response via distinct p110 catalytic subunits. For example, CSF-1 stimulation of macrophages promotes a p110α-dependent mitogenic response, while chemotaxis is mediated by p110β and p105α PI 3-kinase catalytic subunits (71). In another study it was shown that actin reorganization in endothelial cells in response to insulin was dependent on p110β, while platelet-derived growth factor-stimulated actin rearrangements were p110α dependent (72).

These data concur with an emerging model for the regulation of chemotaxis, where the detection, polarization, and movement of a cell toward a chemotactic gradient are controlled by the sequential polarization and activation of key signaling molecules at the leading edge and the posterior and lateral sides of the cell, enabling remodeling of the actin cytoskeleton and ultimately directional cell movement (27, 28, 62, 73). Although much work is required to fully elucidate the molecular mechanisms involved, it is clear that PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinases, PKB (and very likely other PH domain-containing molecules), Rho family GTPases (Rac, Rho, and Cdc42), and the PI 3-kinase pathway.

References