A Pak- and Pix-dependent branch of the SDF-1 α signalling pathway mediates T cell chemotaxis across restrictive barriers

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Pak (p21-activated kinase) serine/threonine kinases have been shown to mediate directional sensing of chemokine gradients. We hypothesized that Pak may also mediate chemokine-induced shape changes, to facilitate leucocyte chemotaxis through restrictive barriers, such as the extracellular matrix. A potent inhibitor, Pakⁱ, was characterized and used to probe the role of Pak-family kinases in SDF-1 α (stromal-cell derived factor-1 α /CXCL12)-induced chemotaxis in a T cell model. Pakⁱ potently inhibited SDF-1 α -induced Pak activation by a bivalent mechanism, as indicated by its complete inactivation upon point mutation of two binding sites, but partial inactivation upon mutation of either site alone. Importantly, Pakⁱ was not toxic to cells over the time frame of our experiments, since it did not substantially affect cell surface expression of CXCR4 (CXC chemokine receptor 4) or integrins, cell cycle progression, or a number of ligand-induced responses. Pakⁱ produced dose-dependent inhibition of SDF-1 α -induced migration through rigid filters bearing small pores;

but unexpectedly, did not substantially affect the magnitude or kinetics of chemotaxis through filters bearing larger pores. SDF-1 α -induced Pak activation was partly dependent on PIX (Pak-interactive exchange factor); correspondingly, an allele of β -PIX that cannot bind Pak inhibited SDF-1 α -induced chemotaxis through small, but not large pores. By contrast, other key players in chemotaxis: G_i, PI3K (phosphoinositide 3-kinase), and the Rho-family G-proteins, Rac and Cdc42 (cell division cycle 42), were required for SDF-1 α -induced migration regardless of the barrier pore-size. These studies have revealed a distinct branch of the SDF-1 α signalling pathway, in which the Rac/Cdc42 effector, Pak, and its partner, PIX, specifically regulate the cellular events required for chemokine-induced migration through restrictive barriers.

Key words: chemotaxis, enzyme inhibitor, p21-activated kinase (Pak), protein kinase, signal transduction, T cell.

INTRODUCTION

Chemokines, such as SDF-1 α (stromal-cell derived factor-1 α /CXCL12), trigger profound, spatially polarized cytoskeletal changes [1,2] that culminate in chemotaxis, a process by which cells follow the gradient of a chemokine to its source. SDF-1 α /CXCL12 is a homeostatic chemokine, expressed primarily in lymph node, lung, liver and bone marrow [3], which directs basal trafficking of leucocytes to these organs [4,5]. In addition to its physiological roles, some types of cancers express the SDF-1 α receptor, CXCR4 (CXC chemokine receptor 4), and tend to metastasize to tissues expressing high levels of SDF-1 α [3,6–9].

Chemokine-induced directional sensing on a flat, unobstructed surface depends on PI3K (phosphoinositide 3-kinase), the product of which, Ptd(3,4,5) P_3 (phosphatidylinositol (3,4,5)-trisphosphate), accumulates at the leading edge of migrating neutrophils [10,11]. A Ptd(3,4,5) P_3 -activated positive feedback loop, which depends on PI3K and on the Rho-family G proteins, Rac and Cdc42 (cell division cycle 42), reinforces directionality [12,13]. In this context, it is intriguing that both Pak1 (p21-activated kinase 1), one of the many effectors of Rac and Cdc42, and PIX (Pak-interactive exchange factor), a Pak-associated, Rac- and Cdc42-specific GEF (guanine nucleotide exchange factor) [14,15], are required for directional sensing in neutrophils [16]. Taken together, the current evidence suggests that directional

sensing by neutrophils is co-ordinately regulated by Rac, Cdc42, Pak, PIX and PI3K.

No less important than directional sensing is chemokine-induced ability of cells to cross restrictive barriers, which would otherwise obstruct chemotaxis *in vivo*. A number of strategies are employed by different cell types to cross such barriers; however, leucocytes tend to rely on an amoeboid strategy, which is a protease-independent mode of chemotaxis, involving dramatic changes in cell shape [17]. To date, the signalling pathways mediating the chemokine-induced, amoeboid-type shape changes that accompany chemotaxis across restrictive barriers have not been well understood.

The archetypal Pak1, 2 and 3 (which in the present study will be collectively referred to as Pak) are an evolutionarily conserved family of serine/threonine kinases, regulated by the Rho-family G-proteins, Rac and Cdc42, and are implicated in the regulation of cytoskeletal architecture and directionally polarized processes in organisms ranging from yeast to man [18,19]. Pak family enzymes have a conserved structure, including a C-terminal serine/threonine kinase domain and an N-terminal regulatory domain (see Figure 1 for details). Their very low basal kinase activity is maintained by an N-terminal auto-inhibitory loop, that is highly conserved in Pak1, 2 and 3, which interacts with the kinase domain and inhibits its function [20–23]. Pak is opened up and activated upon binding of GTP-bound Rac1 or Cdc42 to an

Abbreviations used: Cdc42, cell division cycle 42; CXCR4, CXC chemokine receptor 4; Cy5, Cyanine 5; EGFP, enhanced green fluorescent protein; Erk1/2, extracellular-signal-regulated kinase 1/2; FCS, foetal calf serum; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; KI, kinase inhibitor; Pak, p21-activated kinase; PBD, p21-binding domain; PI3K, phosphoinositide 3-kinase; Ptd(3,4,5) P_3 , phosphatidylinositol (3,4,5)-trisphosphate; PIX, Pak-interactive exchange factor; RPE, R-phycoerythrin; SDF-1 α , stromal-cell derived factor-1 α ; SH3, Src homology 3; TCR, T cell receptor; VLA, very late antigen.

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Figure 1 Domain structure of Pak1 and the Pak-inhibitory constructs used in the present study

Pak^R consists of residues 2–265 of Pak1, whereas Pak^R-PBD-mut incorporates the H83L/H86L mutation that inactivates the PBD [20,22,27]. Pakⁱ consists of residues 83–265 of Pak1 expressed as an N-terminal FLAG-tagged GST fusion protein. A control inhibitor, Pak^{i-2m}, incorporates the L107F mutation, which inactivates the kinase-inhibitory domain [23,53], and the P192G/R193A mutation, which inactivates the PIX-binding site [14,25]. Pak^{i-KI-mut} and Pak^{i-PIX-mut} only incorporate either the first or the second mutation respectively.

N-terminal PBD (p21-binding domain) that partially overlaps with the auto-inhibitory domain [20–22]. In addition, the SH3 (Src homology 3) domain of the GEFs, α - and β -PIX (also known as COOL-2 and COOL-1 respectively), binds with nanomolar affinity to a proline-rich motif that is highly conserved in Pak1, 2 and 3 [14,24]. The interaction of Pak with PIX is required for ligand-induced activation of Pak1 in some contexts [25].

Previous studies demonstrated dramatic effects of over-expressed Pak on cytoskeletal architecture, indicative of a possible involvement in cell motility [20,26–28]. Subsequent studies revealed that Pak is activated in response to a variety of chemokines [16,29–33], whereas a catalytically inactive Pak kinase domain inhibits chemotaxis [31]. In a previous study, a Pak1-specific small interfering RNA was shown to inhibit directional sensing of neutrophils, without affecting chemokine-induced motility itself [16]. Similarly, in rat primary fibroblasts Pak kinase activity is required to control the directional polarity of membrane ruffling in the context of a wound healing assay [34].

In the present study, we found that Pak and PIX are specifically required for migration through small, but not large pores, and therefore probably mediate chemokine-induced amoeboid-type shape changes. By contrast, other signalling elements, such as $G\alpha_i$, PI3K, Rac and Cdc42, are more generally required for chemotaxis, independent of the barrier pore size.

EXPERIMENTAL

Antibodies and reagents

A rabbit polyclonal anti-serum was raised against the Pak1-derived N-terminal peptide, SNNGLDIQDKPPAPPMRNTS, using the Sigma custom antisera service. M2 (an anti-FLAG epitope antibody) and pertussis toxin were purchased from Sigma. The anti-CXCR4 antibody was obtained from Pharmingen (BD Biosciences), anti-VLA (very late antigen) 4 and anti-VLA5 anti-

bodies were obtained from Dako. Surface staining with these antibodies was detected with an RPE (R-phycoerythrin)-Cy5 (Cy-anine 5)-conjugated F(ab')₂ rabbit anti-mouse antibody (Dako). The monoclonal antibody, C305, was used for anti-TCR (T cell receptor) stimulations [35]. SDF-1 α was obtained from R&D systems. Wortmannin was obtained from Calbiochem. The RPE-conjugated anti-H-2K^k (allelic major histocompatibility complex antigen, H-2K) monoclonal antibody and an RPE-conjugated anti-CD69 antibody were obtained from Serotec. Anti-Erk1/2 (extracellular-signal-regulated kinase 1/2) and anti phospho-Erk1/2 (Thr²⁰²/Tyr²⁰⁴) antibodies were purchased from Cell Signaling Technology. The anti phospho-Akt (S473) antibody was obtained from Biosource (Camarillo, CA, U.S.A.). Protein A/G agarose beads were obtained from Santa Cruz.

Plasmids

All expression vectors used in this study were based on pBI-EGFP (enhanced green fluorescent protein) (Clontech/BD Biosciences), which utilizes a bidirectional tetracycline-responsive promoter to drive co-expression of the gene of interest and EGFP in any cell line that stably expresses the tetracycline trans activator [36]. A Kozak sequence, initiation codon, FLAG tag and two unique restriction sites (AscI and ClaI) were inserted into the polylinker of pBI-EGFP, using the appropriate annealed oligonucleotides (details available upon request from D. Yablonski), to create pBI-FLAG. pBI-H-2K^k was derived from pBI-FLAG, by replacing the EGFP coding sequence with a truncated form of the allelic mouse major histocompatibility complex antigen, H-2K^k [37], isolated from the plasmid pMACS K^k.II (Miltenyi Biotec GmbH, Germany). The Pak inhibitory fragments used in this paper are depicted in Figure 1. The following nomenclature is used throughout the present study: x-GFP, denotes the gene 'x', cloned into pBI-FLAG and co-expressed with EGFP, whereas x-K^k denotes coexpression of gene 'x' with H-2K^k. Pak^R includes residues 2–265 of Pak1, which were amplified by PCR, with the concomitant addition of an AscI site at the 5' end and a ClaI site at the 3' end, and cloned into pBI-FLAG, in frame with the N-terminal FLAG tag. Pak^R-PBD-mut is identical to Pak^R, except for the presence of the H83L/H86L mutation, which inactivates the PBD [20,27]. Pakⁱ includes residues 83–265 of Pak1, expressed as a FLAG-tagged, GST (glutathione S-transferase) fusion protein. Standard overlapping PCR was used to amplify the desired wild-type or point-mutated Pak1 fragments, while fusing them in frame to the GST cDNA derived from pGEX-2TK (Amersham), with the concomitant addition of an AscI site at the 5' end and a ClaI site at the 3' end. These were subcloned into the AscI and ClaI sites of pBI-FLAG, or pBI-H-2K^k, in frame with the N-terminal FLAG tag. Myc-tagged Cdc42^{N17} was amplified by PCR, with the concomitant addition of a PvuII site at the 5' end and an NheI site at the 3' end, and cloned into pBI-EGFP. Myc-tagged Rac1^{N17} was cloned into pBI-EGFP by blunt ligation. FLAG-tagged β -PIX^{SH3-mut} (W43P/W44G) [14] was cloned into pBI-EGFP by blunt ligation. All PCR-derived constructs were verified by sequencing.

Cell culture, transfection, and purification of transfected cells

Jurkat VP/tetR cells, expressing the Tet-trans activator, were kindly provided by David Straus (Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA, U.S.A.) [38]. Cells were grown in RPMI medium containing penicillin/streptomycin, glutamine and 5 % FCS (foetal calf serum). For transient transfection of pBI-FLAG-based constructs, 20 $\times$ 10⁶ cells were electroporated with 20 μ g of DNA, in 0.4 ml of preheated (37 °C) RPMI-1640 containing

10 mM dextrose and 0.1 mM dithiothreitol, using Gene Pulser (Bio-Rad Laboratories, Hercules, CA, U.S.A.), at a setting of 234 V and 1000 μ F, using a 0.4 cm preheated cuvette (Bio-Rad). Transfected cells were resuspended in growth medium and were used for experiments 16–22 h after transfection. In experiments using pBI-H-2K^k-based constructs, 20×10^6 cells were electroporated with 15 μ g of DNA at room temperature in 0.3 ml of RPMI-1640 containing 10 % FCS, using an ECM 830 square wave electroporator (BTX, a subdivision of Genetronics, San Diego, CA, U.S.A.) at a setting of 300 V, 10 ms and in LV mode. After 18–24 h, the cells were stained with a PE-conjugated anti-H-2K^k antibody (Serotec), and H-2K^k-positive cells were isolated using the EasySep PE Selection kit (Stem Cell Technologies) according to the manufacturer's instructions. The purified cells were cultured in growth medium for an additional 16–20 h before an experiment.

Pak1 kinase assay

A quantitative immune complex Pak1 kinase assay, using Histone, H4, as a substrate, was carried out essentially as described [39], except that the stimulant was 100 ng/ml SDF-1 α (R&D Systems), and the detergent used for cell lysis was 1 % n-dodecyl- β -D-maltoside (Calbiochem).

Migration assay

Transfected cells were purified by Histopaque 1077 and resuspended at 5×10^6 cells/ml in migration medium [RPMI-1640 containing 5 % FCS, penicillin, streptomycin, glutamine and 10 mM Hepes (pH = 6.9)]. A 100 μ l of cells was loaded in the upper chamber of an uncoated transwell insert (Costar) and 600 μ l of migration medium, with or without SDF-1 α , was added to the lower well. The SDF-1 α concentration was 10 ng/ml, unless otherwise indicated. After 2.5 h at 37 °C in a 5 % CO₂ incubator, cells were collected from the lower chamber, centrifuged at 252 g for 3 min at room temperature, resuspended in FACS buffer with the addition of 250 μ l of fluorescent reference beads (PKH26, Sigma) and counted using a FACS apparatus. Transfected cells were categorized by their expression of EGFP, and the number of migrated cells from each sub-population was normalized to the number of reference beads in each sample. The migration of non-transfected cells was measured essentially as described above, except that cells were pretreated with vehicle or the indicated inhibitor, as described in the legend of Figure 7. Statistical analyses were performed using the non-parametric Kruskal–Wallis test.

Cell cycle

After transient transfection with a pBI-H-2K^k-based plasmid and immunomagnetic purification of transfected cells, 2×10^6 cells were resuspended in 0.5 ml of PBS and added drop-wise to 4.5 ml of ice-cold 70 % ethanol. After 2 h on ice, cells were washed twice and incubated for 30 min at room temperature in 1 ml of 0.1 % Triton, 0.2 mg/ml RNase A and 20 μ g/ml propidium iodide, and incorporation of propidium iodide was measured by FACS in the FL3 channel.

RESULTS

SDF-1 α -induced activation of Pak

SDF-1 α -induced activation of Pak1 was assessed in Jurkat T cells, using a quantitative immune complex kinase assay. SDF-1 α induced a greater than 5-fold activation of Pak1, which peaked at 1 min and returned to basal levels after 5 min (Figure 2). Pak1 was markedly activated by concentrations of SDF-1 α as low as

Figure 2 SDF-1 α -induced activation of endogenous Pak1

Cells were stimulated with 100 ng/ml SDF-1 α for the times indicated, lysed, and anti-Pak1 immune complexes from 3×10^6 cells were assayed for kinase activity, using H4 as a substrate. The results are representative of four independent experiments.

10 ng/ml (results not shown), indicating that this response occurs at physiologically relevant concentrations of chemokine. In the experiments described below, we explored the significance of SDF-1 α -induced Pak activation, using a potent inhibitor of Pak-family enzymes.

Pakⁱ, an inhibitor of ligand-induced Pak activation

We designed an inhibitory fragment, Pakⁱ (Figure 1), derived from the N-terminal regulatory domain of Pak1 that encompasses two functional sites: the KI (kinase inhibitory) segment, which can bind to and inhibit the catalytic domain *in trans* [20–23], and the PIX-binding site, which can bind to PIX with nanomolar affinity [14] and competitively inhibit its binding to cellular Pak. Both sites show greater than 90 % identity with Pak1, 2 and 3 [14,21,22], suggesting that Pakⁱ should inhibit all three enzymes with equal potency. Both sites were inactivated by point mutations in the control inhibitor to give Pak^{i-2m} (Figure 1). We emphasize that Pakⁱ does not include a functional BPD [20,22]; as a result, we expected it to inhibit specifically Pak-family enzymes without inhibiting other Rac and Cdc42 effectors.

Pakⁱ was expressed in Jurkat cells by transient transfection, using a vector (Pakⁱ-K^k) that drives expression of both Pakⁱ and H-2K^k from a bi-directional promoter [36]. We routinely observed approx. 60 % transfection efficiency, as indicated by surface H-2K^k expression (Figure 3A, top panel). H-2K^k-expressing cells were isolated by a rapid immunomagnetic separation procedure, producing a homogeneous population consisting of greater than 96 % transfected cells (Figure 3A, bottom panel). The roughly similar transfection efficiency and viability routinely observed upon transient transfection of Pakⁱ, as compared with the control vector, testify that Pakⁱ is not acutely toxic to cells (results not shown). Consistent with this conclusion, cell cycle analysis of purified Pakⁱ- or vector-transfected cells revealed an identical distribution throughout the phases of the cell cycle, as late as 2 days following transient transfection (Figure 3B).

SDF-1 α -induced activation of endogenous Pak1 was measured in purified, homogeneous populations of cells expressing either Pakⁱ-K^k, Pak^{i-2m}-K^k, or H-2K^k alone. Both basal and SDF-1 α -induced Pak1 activity were profoundly inhibited by Pakⁱ (Figure 3C). Pak^{i-2m}, although expressed at the same level as Pakⁱ (results not shown), was completely inactive, demonstrating that the striking inhibitory activity of Pakⁱ is mediated by the expected mechanisms, which depend on the kinase inhibitory loop and/or the PIX-binding site of Pakⁱ.

Figure 3 Pakⁱ, a specific and efficient inhibitor of ligand-induced Pak1 activation

(A) Surface-expressed H-2K^k was detected by FACS, 24 h after transient transfection with Pakⁱ-K^k (top), or after subsequent immunomagnetic purification of H-2K^k-expressing cells (bottom). The percentage of H-2K^k-expressing cells falling within the range M1 is indicated. (B) Cells transiently transfected with Pakⁱ-K^k (bold line) or H-2K^k alone (grey line) were purified as in (A) and the following day the cell cycle distribution was measured by FACS analysis of propidium iodide-labelled cells. The results are representative of four independent experiments. (C) Cells transfected with the indicated constructs were purified as in (A). After 18 h, the cells were stimulated for 1 min with 100 ng/ml SDF-1 α , or were mock stimulated, and the activity of endogenous Pak1 was measured as in Figure 2. The kinase assay was performed in triplicate, error bars indicate the standard deviation, and one of three representative experiments is shown. (D) Cells transiently transfected with Pakⁱ-K^k or H-2K^k alone were purified as in (A), and the following day were stimulated for 2 min with 100 ng/ml SDF-1 α , or were mock stimulated, and Erk activation was measured by Western blotting with an anti-phospho-Erk1/2 antibody (top). The same blot was stripped and reprobed with the anti-Erk1/2 antibody (bottom). The results are representative of three independent experiments. (E) After 24 h of transient transfection with Pakⁱ-GFP or GFP alone, cells were stimulated overnight with a plate-bound anti-TCR antibody, or were mock stimulated, and were then stained with a PE-Cy5-conjugated-anti-CD69 antibody. FACS was used to gate GFP high-expressing cells, which were then analysed for CD69 expression. Dotted line: vector, unstimulated; thin line: Pakⁱ, unstimulated; shaded area: vector, anti-TCR antibody; bold line: Pakⁱ, anti-TCR antibody.

The potency of Pakⁱ, as an inhibitor of ligand-induced Pak activation, cannot be attributed to general toxicity, since other complex signalling events can occur normally in Pakⁱ-expressing cells. For example, SDF-1 α -induced down-regulation of CXCR4 was not affected by Pakⁱ (results not shown), whereas SDF-1 α -induced Erk1/2 activation was only moderately decreased (Figure 3D). TCR-induced upregulation of CD69 was also not affected by Pakⁱ (Figure 3E). Taken together, the above results suggest that Pakⁱ is a potent, but highly specific reagent, which can be used to identify particular ligand-induced events that depend upon Pak activation.

Efficient, SDF-1 α -induced transwell chemotaxis depends upon Pak activation

To explore the role of Pak catalytic activity in SDF-1 α -induced chemotaxis, Jurkat cells were transiently transfected with Pakⁱ-GFP, in which EGFP serves as a marker of transfected cells. Transient transfection with Pakⁱ-GFP produced a heterogeneous population, consisting of non-expressing, low-, medium- and

high-expressing cells (Figure 4A). This unsorted mixture of cells was loaded into the upper well of a transwell apparatus, and allowed to migrate, under the influence of SDF-1 α into the lower chamber, through a polycarbonate filter bearing 5 μ m diameter pores.

Post-experimental FACS analysis was performed to measure the percentage of cells in each expression category that migrated to the lower chamber. Migration was clearly SDF-1 α dependent (Figure 4B). Under the influence of SDF-1 α , Pakⁱ-GFP-transfected cells exhibited an inverse correlation between the level of expression of EGFP and the percentage of cells that successfully crossed the membrane; by contrast, the inactive, double-mutated inhibitor, Pakⁱ-2m, did not affect migration at any dose (Figure 4B). Since EGFP expression from this vector is proportional to the expression of Pakⁱ [36], this result indicates that Pakⁱ inhibits SDF-1 α induced chemotaxis in a dose-dependent manner.

To rule out trivial reasons for the Pakⁱ-mediated reduction in SDF-1 α -induced chemotaxis, we tested whether Pakⁱ/GFP affects the expression of key cell surface molecules that are relevant to chemotaxis. By gating for intensely green fluorescent cells

Figure 4 Dose-dependent inhibition of SDF-1 α -induced migration by Pakⁱ

(A) A typical distribution of EGFP expression after transient transfection of Pakⁱ-GFP (bold line) compared with the autofluorescence from untransfected cells (thin line). The indicated gates were used to represent untransfected (M1) low- (M2) medium- (M3) and high-expressing (M4) cells in this and subsequent experiments. (B) After 20 h of transient transfection with the indicated constructs, the mixed, unsorted population, as shown in (A), was loaded into the upper chamber of a transwell insert bearing 5 μ m diameter pores, in the presence or absence of 10 ng/ml SDF-1 α in the lower chamber, as indicated. The percentage migration to the lower chamber was calculated separately for each expression category. A representative experiment is shown; additional repeats of this experiment, including error bars, can be seen as part of Figure 5(B). (C)–(E) After 24 h of transient transfection with Pakⁱ-GFP (bold line) or GFP alone (thin line), cells were stained with antibodies to CXCR4 (C), VLA-4 (D), or VLA-5 (E), followed by an RPE-Cy5-conjugated secondary antibody. FACS was used to gate the upper 1.5 log of EGFP-expressing cells, which were then analysed for surface antigen expression. Dotted line: control staining of untransfected cells with secondary antibody alone, or isotype control. The results are representative of three independent experiments.

(which also express the highest level of Pakⁱ), we found that high levels of Pakⁱ did not decrease the expression of CXCR4, VLA4 or VLA5, as compared with vector-transfected cells within the same gate (Figure 4C–4E).

An interaction between Pak and PIX is required for SDF-1 α -induced Pak activation and for SDF-1 α -induced migration

Pak binds with nanomolar affinity to the Dbl-family GEFs, α - or β -PIX [14]. A variant of Pakⁱ, in which the KI domain is mutated, but the PIX-binding site is intact (Pak^{i-KI-mut}, see Figure 1), was used to interfere specifically with the binding of PIX to Pak. Although less potent than Pakⁱ, Pak^{i-KI-mut} markedly inhibited SDF-1 α -induced activation of Pak1 (Figure 5A), suggesting that an interaction with PIX is required for optimal Pak activation in the context of the SDF-1 α signalling pathway.

The reverse experiment was performed using the inhibitor Pak^{i-PIX-mut} (Figure 1), in which the PIX-binding site is mutated, but the kinase-inhibitory domain remains intact. Similar to Pak^{i-KI-mut}, Pak^{i-PIX-mut} markedly inhibited SDF-1 α -induced activation of Pak1 (Figure 5A), but was less potent than Pakⁱ. Thus the potency of Pakⁱ appears to result from a bivalent mechanism of action: it inhibits the interaction of Pak with PIX, and also directly binds to and inhibits the catalytic cleft of Pak.

Both Pak^{i-KI-mut} and Pak^{i-PIX-mut} inhibited SDF-1 α -induced migration in cells expressing a high level of the inhibitor, but neither mutant substantially inhibited migration at low or medium expression levels (Figure 5B), consistent with their decreased

potency as inhibitors of Pak (Figure 5A). These results demonstrate a correlation between the degree of inhibition of Pak1 kinase activity and the ability to inhibit SDF-1 α -induced migration at relatively low expression levels.

Pak activity is specifically required for SDF-1 α -induced migration through restrictive barriers

Among other parameters, the transwell assay measures the ability of cells to pass through restrictive pores that are smaller than the resting cell diameter under the influence of a chemokine. Since Pak is strongly implicated in the regulation of cytoskeletal architecture, we hypothesized that Pak may mediate SDF-1 α -induced shape changes that are required for passage through small pores.

To test this notion, we compared the effect of Pakⁱ on SDF-1 α -induced transwell migration through pores of different diameters: 3, 5 and 8 μ m. The absolute rate of migration differed depending on pore size, reflecting the intuitive notion that a large pore is easier to pass through, but in all cases migration was markedly SDF-1 α -dependent, indicating that cells must receive a signal to migrate, and in no case are cells merely 'falling through' the pores (Figure 6A, right panel, and results not shown). We found the effect of Pakⁱ on SDF-1 α -induced migration to be markedly pore size-dependent. Whereas Pakⁱ high-expressing cells barely migrated through 3 μ m diameter pores (Figure 6A, left panel), they migrated through 5 μ m diameter pores at approx. 50 % of the rate of non-expressing cells (Figure 6A, middle panel) and

Figure 5 PIX contributes to SDF-1 α -induced Pak1 activation

(A) Cells transiently transfected with the indicated derivatives of Pakⁱ-K^k were purified as described in Figure 3(A) and the following day SDF-1 α -induced activation of endogenous Pak1 was measured as in Figure 3(C). The kinase assay was performed in triplicate, with error bars indicating the standard deviation, and one of four representative experiments is shown. Lower panel: cell lysates were separated by SDS/PAGE, and expression of the Pakⁱ constructs was detected using an anti-FLAG antibody. (B) Cells were transiently transfected with vector or the indicated derivatives of Pakⁱ-GFP, and SDF-1 α -induced migration was measured using 5 μ m diameter pore transwells as in Figure 4(B). To correct for variability in the absolute rates of migration between independent experiments, we considered the migration of non-expressing cells as an internal control to which the migration of the other populations was compared. The migration of GFP-expressing cells from each of the categories depicted in Figure 4(A) was normalized to the migration of non-expressing cells that were from the same transfection and were migrating in the same well, and then multiplied by 100 to yield relative migration. Results are the average of five independent experiments, error bars indicate the standard deviation. Lower panel, expression of the Pakⁱ constructs from a representative experiment was detected as in (A). kDa.

migrated through 8 μ m diameter pores at approx. 80 % of the rate of non-expressing cells (Figure 6A, right panel), at every time point tested. At the 2.5 h time point, Pakⁱ barely affected SDF-1 α -induced migration through 8 μ m diameter pores, whereas an approx. 50 % inhibition was observed through 5 μ m diameter pores and 75 % inhibition was observed through 3 μ m diameter pores (Figure 6B). We repeated the experiment at 5-fold lower and 5-fold higher concentrations of SDF-1 α . In all cases, Pakⁱ produced 70–80 % inhibition of migration through 3 μ m diameter pores, but less than 15 % inhibition of migration through 8 μ m diameter pores (Figure 6C).

Using a different experimental system, Li et al. [16] have reported that Pak activity is required for directional sensing, but not for chemokine-induced motility itself (chemokinesis). To assess the potential contribution from chemokinesis within our

experimental system, we added an equal concentration of SDF-1 α to both the upper and lower chambers of the transwell apparatus. Under these conditions, there is no chemotactic gradient, and migration to the lower well occurs by chemokinesis. We found that the rate of chemokinesis through all pore sizes was relatively low, whereas a several-fold higher level of migration occurred when SDF-1 α was added to the lower well alone, to create a chemotactic gradient (Figure 6D, compare white with grey bars). Strikingly, both chemokinesis and chemotaxis through 3 μ m diameter pores was inhibited by Pakⁱ, but neither was inhibited through 8 μ m diameter pores (Figure 6D). These results demonstrate that when SDF-1 α was added to the lower well alone, there was no substantial erosion of the gradient over the time course of our experiment, such that chemotaxis predominates, regardless of the pore size. Furthermore, Pak activity is required for migration through small, but not large pores, regardless of the presence of a chemotactic gradient.

We next validated the above result, using a different reagent to inhibit the Pak pathway. To this end, we expressed a dominant negative form of β -PIX, incapable of binding Pak due to an inactivating mutation in its SH3 domain [14]. β -PIX^{SH3-mut} expression resulted in a pore size-dependent migration defect, which was apparent in high-expressing cells (Figure 6E) and resembled the defect seen upon the inhibition of Pak (compare Figures 6B and 6E). Taken together these results suggest that Pak and PIX co-operatively regulate the signalling events required for SDF-1 α -induced migration through small pores.

In contrast to Pakⁱ and β -PIX^{SH3-mut}, which specifically inhibited migration through small pores, pertussis toxin, which inactivates G α_i , inhibited migration regardless of the pore size (Figure 7A). Similarly, wortmannin, a specific inhibitor of PI3K, produced a dose-dependent but pore-size-independent inhibition of SDF-1 α -induced chemotaxis (Figure 7B). Thus different inhibitors of chemotaxis differ with respect to their effects on chemotaxis through large versus small pores. Whereas G α_i and PI3K appear to represent the basic SDF-1 α sensing or responsive machinery, the Pak–PIX complex appears to represent a particular branch of the chemokine-induced signalling network that is specifically required to pass through restrictive barriers.

A Pak-dependent branch of the SDF- α signalling pathway, specifically required for migration through restrictive barriers, diverges from the general migration pathway downstream of Rac1 and Cdc42

To determine where the pore-size-dependent pathway diverges from the general chemotactic pathway, we inhibited the upstream activators of Pak, Rac and Cdc42, using another Pak-derived inhibitor, Pak^R [39,40]. Encompassing the entire N-terminal regulatory domain of Pak1 (Figure 1), Pak^R includes the PBD, which can bind to activated Rac and Cdc42 [20,22,27], and thereby inhibit all Rac and Cdc42-dependent signalling events, including, but not limited to the activation of Pak1. Pak^R markedly inhibited migration through 8 μ m diameter pores (> 70 % inhibition), and this effect was abolished upon mutation of the PBD (Figure 8A), suggesting that Rac and/or Cdc42 is required for migration regardless of the pore size. Strikingly, Pak^{R-PBD-mut}, which retains the Pak-inhibiting regions found in Pakⁱ but cannot bind to Rac or Cdc42 [20,22,27], produced pore size-dependent inhibition of migration, virtually identical to that seen using Pakⁱ (compare Figures 8A and 6B). To distinguish further the requirement for Pak from that of other Rac or Cdc42 effector proteins, dominant negative forms of these small G proteins were expressed in Jurkat cells. Rac1^{N17} almost completely abolished cell migration in high-expressing cells and decreased it by approx.

Figure 6 Pak and PIX are required for SDF-1 α -induced migration through small, but not large pores

(A) After 20 h of transfection with Pak1-GFP, the heterogeneous population, including both expressing and non-expressing cells, was loaded into transwells bearing 3 μ m (left), 5 μ m (middle), or 8 μ m (right) diameter pores, and migration was allowed to proceed for the indicated time in the presence or absence of 10 ng/ml SDF-1 α in the lower chamber, as indicated. The percentage migration of non-expressing (filled symbols) or high-expressing (open symbols) cells is shown. This experiment is representative of three experiments that produced similar results. (B) SDF-1 α -induced migration of Pak1-GFP-transfected cells was measured as in (A), except that cells were collected only at the 2.5 h time-point. The migration of high-expressing cells was normalized to the migration of non-expressing cells from the same transfection and that were migrating in the same well, and then multiplied by 100 to yield relative migration. Results are the average of five independent experiments; error bars indicate the standard deviation. The differences between the three groups were statistically significant ($P = 0.002$). (C) The relative, SDF-1 α -induced migration of Pak1-GFP-transfected cells was assessed as in (B), except that different concentrations of SDF-1 α were added to the lower chamber, as indicated. (D) After 20 h of transfection with Pak1-GFP, the heterogeneous population was loaded into the upper chamber of transwells of the indicated pore size, in the presence of 10 ng/ml SDF-1 α added to the lower chamber alone (chemotaxis) or both the lower and upper chambers (chemokinesis). The percentage migration of non-expressing or high-expressing cells to the lower chamber was assessed after 2.5 h. Results are the average of three independent experiments; error bars indicate the standard deviation. (E) Cells were transiently transfected with β -PIX^{SH3-mut}-GFP, and the relative SDF-1 α -induced migration of high-expressing cells was measured through transwells bearing pores of the indicated diameter, as in (B). Results are the average of four independent experiments; error bars indicate the standard deviation.

65 % in medium-expressing cells, regardless of the pore size (Figure 8B). Similarly, Cdc42^{N17} inhibited Jurkat cell migration but to a lesser extent, by approx. 60 % in high-expressing cells, regardless of the pore size (Figure 8C). Thus both Rac1 and Cdc42 are required for chemotaxis independently of the pore size, whereas one of their downstream effectors, Pak, is primarily required for chemotaxis through small pores.

DISCUSSION

The Pak family of serine-threonine kinases has been strongly implicated in the regulation of cytoskeletal architecture and in the

regulation of cellular directionality [16,34]. Using an antisense strategy to decrease the expression of Pak1, Li et al. [16] showed that Pak1 is specifically required for directional sensing in neutrophils. Although decreased expression of Pak1 did not affect chemokinesis on a flat, unobstructed surface, the ability of cells to follow a gradient of chemokine across this surface was severely impaired [16]. In the present study, we have expanded on the findings of Li et al. [16] by demonstrating a second role played by Pak-family kinases in the context of chemotaxis: we show that Pak, and the associated PIX, are required for lymphocytes to pass through restrictive openings under the influence of a chemokine. Whereas the assay used by Li et al. [16] is particularly well suited to measuring directional sensing, the transwell assay we

Figure 7 Other signalling molecules required for migration independently of pore size

(A) Cells were pre-treated for 30 min at 37 °C with 100 ng/ml pertussis toxin and migration was measured as in Figure 6(B). Migration of inhibitor-treated cells was normalized to the migration of vehicle-treated cells, migrating in parallel wells of the same pore size, and then multiplied by 100 to yield relative migration. Results are the average of three independent experiments; error bars indicate the standard deviation. (B) Cells were pre-treated for 1.5 h at 37 °C with the indicated concentration of wortmannin, allowed to migrate in the continued presence of wortmannin, and relative migration was measured as in (A). Results are the average of 4 (100 nM) or 5 (200 nM) independent experiments; error bars indicate the standard deviation. To assess the degree of inhibition of PI3K, total cell lysates from a representative experiment were probed with anti-phospho-Akt (bottom panel).

have used cannot measure directionality because cells can only move downward within this apparatus. By contrast, by the use of transwells bearing different size pores, this assay can measure the ability of cells to move chemotactically through restrictive barriers.

Within the intact organism, cells utilize a variety of strategies to traverse restrictive barriers such as the extracellular matrix as they follow chemotactic gradients. Fibroblasts and some types of cancer cells tend to migrate in a mesenchymal fashion, using pericellular proteases to degrade the extracellular matrix and forge a path along which the cell can migrate [41]. By contrast, leucocytes tend to migrate in a protease-independent, amoeboid fashion [42]. The physiological and pathological importance of amoeboid-type migration is exemplified by the ability of some cancer cells, upon inhibition of metalloproteinases, to undergo a mesenchymal-amoeboid transition, facilitating continued protease-independent tumour dissemination [43].

Figure 8 Rac1 and Cdc42 are required for migration independently of the pore size

(A) Cells were transiently transfected with Pak^R-GFP, which encompasses the entire N-terminal regulatory domain of Pak1, or with Pak^{R-PBD-mut}-GFP, bearing an inactivating mutation in the PBD. The relative SDF-1 α -induced migration of high-expressing cells was measured through transwells bearing pores of the indicated diameter, as in Figure 6(B). This experiment is representative of two that showed the same result. (B) Cells were transiently transfected with Rac1^{N17}-GFP, and SDF-1 α -induced migration was measured through transwells bearing pores of the indicated diameters. The relative migration of medium- and high-expressing cells is shown. Results are the average of 3 independent experiments, error bars indicate the standard deviation. (C) Cells were transiently transfected with Cdc42^{N17}-GFP, and the relative SDF-1 α -induced migration of high-expressing cells was measured through transwells bearing pores of the indicated diameter, as in Figure 6(B). Results are the average of three independent experiments; error bars indicate the standard deviation.

In the present study, we have identified two signalling elements, Pak and PIX, that are specifically required for SDF-1 α -induced T cell chemotaxis across restrictive barriers. Pak-inhibited cells clearly sense the presence of SDF-1 α , as reflected by a decreased, but nonetheless marked SDF-1 α -induced activation of Erk1/2, and only moderately decreased migration through large (8 μm diameter) pores. By contrast, Pakⁱ is a potent and dose-dependent inhibitor of chemokine-induced migration through small pores (3 or 5 μm diameter). Similar, pore size-dependent inhibition of

migration is observed upon inhibition of the Pak–PIX interaction. Consistent with this result, PIX contributes to SDF-1 α -induced activation of Pak1. In contrast to Pak and PIX, the heterotrimeric pertussis toxin-sensitive G protein, G_i, is required for migration through both small and large pores, consistent with its role as a mediator of many of the earliest chemokine-induced signalling events [44]. Similarly, PI3K, a well established mediator of chemotaxis [45,46] is required to an equal degree for migration through both small and large pores. These results, for the first time delineate a particular branch of the chemokine signalling pathway, involving Pak and PIX, which is dispensable for sensing the chemokine signal but is specifically required for migration across rigid, restrictive barriers. Further research may uncover the cellular or molecular targets of Pak, which facilitate migration through restrictive pores; nonetheless, it seems reasonable to propose that Pak may regulate cytoskeletal or nuclear flexibility, thereby enabling cells to pass through rigid pores that are smaller than the resting cell diameter.

In considering the complex signalling pathways that mediate chemotaxis, it is important to realise that Pak-family kinases represent only one of more than 10 different types of effectors, activated downstream of the Rho-family G proteins, Cdc42 and Rac [47]. Indeed, the physiological role of Pak has been controversial, with some studies demonstrating that Pak is dispensable for Rac- and Cdc42-mediated cytoskeletal changes and cell transformation [48–50], while many other studies convincingly connect Pak to these processes [19,51].

To distinguish the particular contribution of Pak-family kinases from the composite contribution of all Rac and Cdc42 effectors, we compared Pakⁱ, with Pak^R, and with Pak^{R-PBD-mut}, which bears an inactivating mutation in the PBD. By virtue of its functional p21-binding site, Pak^R can bind to activated Rac and Cdc42, thereby blocking their interactions with all downstream effectors, including, but not limited to Pak-family kinases. Strikingly, Pak^R inhibits migration through all pore sizes, whereas Pak^{R-PBD-mut}, like Pakⁱ, inhibits migration in a pore size-dependent manner. Likewise, cells expressing dominant negative forms of Rac or Cdc42 exhibit a pore size-independent inhibition of migration. We conclude that the small G proteins, Rac and Cdc42, are required for chemokine-induced migration itself, whereas a pore-size-specific Pak-dependent migration pathway diverges from the more general migration pathway immediately downstream of these G proteins. We further suggest that the complex process of chemokine-induced migration is co-ordinately regulated by two or more downstream effectors of Rac and/or Cdc42, with Pak mediating specific processes required for migration through small pores, and other effectors mediating more general migratory processes.

Pak-family kinases are well known to mediate cytoskeletal changes, but some controversy has surrounded the functional importance of their catalytic activity, as opposed to adaptor functions mediated by the N-terminal regulatory domain. Whereas some aspects of migratory behaviour, such as lamellipodia formation, are reportedly triggered by Pak independently of its kinase activity, other aspects, such as persistent directional migration and the maintenance of a single leading edge were found to be kinase dependent [20,28].

We explored this issue using a specific inhibitor, Pakⁱ, which potentially inhibits SDF-1 α -induced activation of Pak1 by a two-fold mechanism that is dependent upon the kinase inhibitory segment and the PIX-binding site. Based on the high degree of sequence conservation between Pak1, 2 and 3, Pakⁱ should inhibit all three enzymes with equal potency, an important characteristic in light of the proven ability of chemokines to activate both Pak1 and Pak2 [29,30], both of which are expressed in Jurkat cells and in human T cells (results not shown). Thus Pakⁱ can be used to probe

all physiological functions of Pak1, 2 and 3, whether these are isotype-specific or redundant.

By comparing Pakⁱ with its mutant derivatives, we observed a clear correlation between the degree of inhibition of Pak1 kinase activity, and the degree of inhibition of migration. Indeed, Pak^{i-PIX-mut}, which bears a mutated PIX-binding site and acts solely by inhibiting the catalytic domain of Pak, induced pore size-dependent inhibition of migration (results not shown). Thus migration through restrictive barriers clearly depends upon the kinase activity of Pak and not merely on its adaptor functions.

Based on our data, we cannot yet conclude whether PIX functions primarily as an adaptor, to recruit Pak to its site of action, or whether it functions as a Pak-specific GEF for Rho-family G proteins. At this point, our results are somewhat supportive of the first possibility, since mutated forms of α - or β -PIX, bearing an inactivating mutation in the Dbl homology domain [14], were less potent inhibitors of migration as compared with β -PIX bearing an inactivated SH3 domain (results not shown).

The involvement of Pak and Pix in mediating migration through restrictive barriers adds an important new aspect to their previously defined involvement in determining the directionality of cell migration [16,34]. Indeed, Pak is often overexpressed in cancer cells, and its activity appears to correlate with the degree of invasiveness [51]. Our results may provide a mechanistic explanation for this finding, since a high level of Pak activity may help cancer cells to traverse restrictive barriers. In addition, Pak activation may facilitate the cellular infiltration that often accompanies inflammation. Inflammation is associated with increased permeability of the vascular endothelium mediated by contraction of endothelial cells, which according to a recent report is mediated by Pak kinase activity [52]. The potential dual role of Pak in mediating inflammatory cellular infiltration, both as a mediator of vascular permeability and as a regulator of the ability of cells to pass through pores of various sizes, suggests that Pak may be an important future therapeutic target in the control of local inflammation.

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