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Biochemistry. Author manuscript; available in PMC 2009 April 13.

Published in final edited form as:

Biochemistry. 2002 June 4; 41(22): 7100–7107. doi:10.1021/bi025902m.

PAK1 Kinase Is Required for CXCL1-Induced Chemotaxis[†]

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Abstract

The CXC subfamily of chemokines plays an important role in diverse processes, including inflammation, wound healing, growth regulation, angiogenesis, and tumorigenesis. The CXC chemokine CXCL1, or MGSA/GROα, is traditionally considered to be responsible for attracting leukocytes into sites of inflammation. To better understand the molecular mechanisms by which CXCL1 induces CXCR2-mediated chemotaxis, the signal transduction components involved in CXCL1-induced chemotaxis were examined. It is shown here that CXCL1 induces cdc42 and PAK1 activation in CXCR2-expressing HEK293 cells. Activation of the cdc42-PAK1 cascade is required for CXCL1-induced chemotaxis but not for CXCL1-induced intracellular Ca^{2+} mobilization. Moreover, CXCL1 activation of PAK1 is independent of ERK1/2 activation, a conclusion based on the observations that the inhibition of MEK-ERK activation by expression of dominant negative ERK or by the MEK inhibitor, PD98059, has no effect on CXCL1-induced PAK1 activation or CXCL1-induced chemotaxis.

CXC chemokines[1] are crucial for the timely recruiting of specific populations of leukocytes to sites of tissue damage during the inflammatory responses. These chemokines are also important in angiogenesis, tumor formation, and tumor metastasis (1–6). In this subfamily, ELR-CXC chemokines with the amino acid sequence glutamic acid–leucine–arginine (the ELR motif) at the N-terminal domain of the ligands, including CXCL1 (melanoma growth stimulatory activity/growth regulated protein, MGSA/GRO), CXCL5 (epithelial-derived neutrophil-activating peptide 78, ENA-78), CXCR6 (granulocyte chemotactic protein-2, GCP-2), and CXCL8 (interleukin-8), are all neutrophil-activating CXC chemokines, which bind to the CXCR1, CXCR2 (CXC chemokine receptor 1 or 2), and/or Kaposi's sarcoma human herpes virus 8 G protein-coupled receptor (1). CXCL1–3 and 5–8 bind to CXCR2 with high affinity, whereas CXCL6 and CXCL8 also bind CXCR1 with high affinity.

[†]We are indebted to the NIH for support through Grants CA34590 (A.R.) and CA56704 (A.R.), to the Vanderbilt Ingram Cancer Center for Grant CA68485, and to the Department of Veterans Affairs for a Merit Award (A.R.), a Career Scientist Award (A.R.), and the GRECC Pilot Project (A.R.).

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[1]Abbreviations: CXC, chemokine, chemokine with the first two conserved cysteine residues separated by an intervening amino acid; DMEM, Dulbecco's modified Eagle's medium; CXCL1 or MGSA/GRO, melanoma growth-stimulatory activity/growth-regulated protein; PAKs, p21-activated kinases; MBP, myelin basic protein; MAP, mitogen-activated protein; MEK, MAP kinase kinase; PBD, p21 binding domain.

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Our earlier studies demonstrated that CXCL1 induces activation of the transcription factor NF-κB through a Ras-MEKK1-MEK4/6-p38 MAP kinase cascade in melanocytes (7). This pathway is involved in CXCL1-induced melanocyte transformation (6). Activation of the phospholipase C–β/PKC/IP3 cascade is required for the CXC chemokine-induced intracellular calcium mobilization in neutrophils (8). Although the chemotactic response to CXCL1 and CXCL8 is well characterized, the signal transduction pathways for the chemotactic responses have not been fully elucidated.

The activated GTPases interact with specific targets that serve as effectors to regulate downstream signaling cascades. The Rho GTPase subfamily, including RhoA, RhoB, RhoC, Rac, and cdc42, has been implicated in the regulation of diverse cellular functions, including actin cytoskeletal dynamics, oxidant generation, transformation, membrane trafficking, apoptosis, transcription, and cell cycle control (9–12). Rac and cdc42 appear to be critical downstream components for the classic chemoattractant fMet-Leu-Phe (13–14). Significant Rac/cdc42 targets are the p21-activated kinases (PAKs).

PAKs play an important role in diverse cellular processes, including cytoskeletal rearrangements (15–19), growth, and apoptosis (20–22). PAKs are Ser/Thr protein kinases, which contain a p21 binding domain (PDB). PAK1 undergoes autophosphorylation and activation upon interacting with the active forms of the small GTPase (p21) Rac or Cdc42 (23). PAK activation is regulated by a variety of external stimuli that act through cell surface receptors, including G protein-coupled receptors (24), growth factor receptor tyrosine kinases (25), proinflammatory cytokine receptors (26), Fc receptors (27), and integrins (28–29). Moreover, a variety of chemoattractants induce rapid activation of PAKs (30). However, the role of PAK1 in chemokine gradient-directed cell movement (chemotaxis) has not been clearly delineated.

Mitogen-activated protein (MAP) kinases represent a point of convergence for cell surface signals regulating cell growth and division. MAP kinases are serine/threonine protein kinases. One member of the MAP kinase family is extra-cellular signal-related protein kinase (ERK). ERK is phosphorylated and activated by MAP kinase kinase (MEK1) (31), which in turn is phosphorylated and activated by the Raf (32). CXCL8 has also been demonstrated to activate the PI3-kinase/Ras/Raf cascade in neutrophils (33). Similarly, CXCL1 induces the activation of ERK through Ras/Raf1 dependent or independent pathways (34). However, it remains controversial whether ERK activation is required for the CXC ligand-induced chemotaxis (33,35). Van Lint et al. reported that ERK activation is involved in IL-8-induced chemotaxis in neutrophils (35). However, Knall et al. reported that the regulation of cell migration by IL-8 is independent of ERK kinase and ERK activation because the ERK kinase inhibitor PD098059 had no effect on IL-8-induced cell migration of human neutrophils (33).

To determine what signal transducers are involved in CXCL1-induced chemotaxis, we used the HEK293 and RBL systems, which provide cellular models to characterize the signaling mechanisms of CXCR2, as such studies are notoriously difficult to perform in primary neutrophils, which express multiple chemokine receptors. Our findings demonstrate that CXCL1 induces PAK1 activation through cdc42. This cdc42–PAK1 cascade is required for CXCL1-induced chemotaxis. In contrast, we demonstrate that the CXCL1 induction of MEK-ERK1/2 is not involved in the CXCL1-induced chemotaxis. Moreover, cdc42–PAK1 and ERK are not required for the intracellular Ca^{2+} mobilization induced by CXCL1.

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EXPERIMENTAL PROCEDURES

Cell Culture

Human embryonic kidney 293 cells (HEK293) were cultured in DMEM supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin, 3 mM glutamine, and 5% heat-inactivated fetal bovine serum (GIBCO BRL, Rockville, MD). The CXCR2-expressing HEK293 polyclonal cells were cultured in the same medium supplemented with 800 μg/mL G418 (Sigma, St. Louis, MO) as previously described (36). The expression level of CXCR2 receptor in the HEK293 cells has been previously verified (36). RBL-2H3 cells and CXCR2-expressing RBL stable clone cells were gifts from Dr. Ricardo Richardson. RBL-2H3 cells were cultured in DMEM supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin, 3 mM glutamine, 15% heat-inactivated fetal bovine serum (GIBCO BRL, Rockville, MD). CXCR2-expressing RBL were cultured in the same medium supplemented with 1000 μg/mL G418 (Sigma) as previously described. The expression level of CXCR2 receptor in the RBL-2H3 cells has been previously verified (37). Purified recombinant human CXCL1 (a kind gift of Repligen Corp., Needham, MA) was used at 50 ng/mL. MEK kinase inhibitor, PD98059 (Calbiochem, La Jolla, CA), was added at the indicated concentration overnight prior to stimulation with CXCL1.

Transfections

CXCR2-expressing HEK293 cells cultured to 80% confluence were transiently transfected with either the empty expression vector, the dominant negative PAK1 (232 K/A) plasmid (a gift from Dr. Jeffrey Frost) (38), dominant negative cdc42, or the dominant negative ERK plasmid (a gift from Dr. Melanie Cobb), using the Lipo-fectAMINE PLUS reagent (GIBCO BRL) according to the manufacturer's protocol. RBL cells (10^7 cells) were transiently cotransfected with CXCR2 receptor (20 μg) and either the empty expression vector (20 μg) or the dominant negative PAK1 (232 K/A) plasmid (20 μg), using electroporation (37). We routinely achieved a transfection efficiency of ~80% with these procedures.

Whole Cell Extracts and Western Blot

Whole cell extracts were prepared from CXCR2-expressing HEK293 treated with CXCL1 for the indicated time after serum starvation for 14 h. Western blots were performed following protocols provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The cells were washed at 4 °C with 1× PBS and lysed in 0.6 mL of RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitor cocktail tablets (Boehringer Mannheim Corp., Indianapolis, IN) and 0.2 mM sodium orthovanadate. Fifty micrograms of soluble protein was boiled, subjected to electrophoresis on a 10% SDS–PAGE reducing gel, and then electrophoretically transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% dry milk in TBS-T buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.05% Tween-20) for 1 h and then incubated for 12–16 h at 4 °C in a 1:1000 dilution (0.2 μg/mL) of the anti-Cdc42 antibody (Santa Cruz Biotechnology, Inc.) and anti-ERK1/2 or the anti-phospho-ERK1/2 (Santa Cruz Biotechnology, Inc.) in TBS-T buffer containing 5% dry milk. After three washings with TBS-T buffer, the membrane was incubated in a 1:3000 dilution of the appropriate anti-mouse or anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Boehringer Mannheim Corp.) in TBS-T buffer with 5% dry milk for 1 h at room temperature. After three washings with TBS-T buffer, the protein bands were detected with the ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. The blots were stripped and reprobed with anti-ERK2.

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Immune Complex Kinase Assays

Whole cell extracts were prepared from CXCR2-expressing HEK293 or CXCR2-expressing RBL-2H3 cells treated with CXCL1 after overnight serum starvation. PAK1 kinase assays were performed as described in the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). Four hundred micrograms of protein of each whole cell extract was immunoprecipitated with 1 μg of PAK1 antibody. Immunoprecipitated PAK1 activity was assayed using the PAK1 substrate myelin basic protein (MBP) (Sigma). Kinase reactions were initiated by addition of 2 μg of MBP and kinase buffer containing 500 μM cold ATP and 10 μCi of [γ-^{32}P]ATP. Reactions were incubated for 30 min at 30 °C and terminated by the addition of an equal volume of 2 × SDS loading buffer followed by boiling for 5 min. Phosphorylated proteins were resolved on a 10% SDS–PAGE reducing gel and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). The phosphorylated bands were visualized by autoradiography. The blot was probed with PAK1 antibody to monitor equal loading of PAK1.

Chemotaxis Assay

Chemotaxis assays were performed on the HEK293 or the RBL-2H3 cells transiently cotransfected with CXCR2 and other constructs as described previously (36,37). Briefly, a 96-well chemotaxis chamber (Neuroprobe Inc., Gaithersburg, MD) was used, and the lower compartment of the chamber was loaded with 450 mL of chemotaxis buffer (1 mg/mL ovalbumin/DMEM) containing CXCL1 diluted at the indicated concentration in the chemotaxis buffer. Polycarbonate membranes (10- or 8-μm pore size) were coated on both sides with 20 μg/mL human collagen type IV (Sigma) and incubated for 2 h at 37 °C. The cells were removed from the plate by trypsinization and incubated in 5% FBS/DMEM for 2 h at 37 °C to allow restoration of receptor expression. The cells were washed with chemotaxis buffer and then loaded into the upper chamber in 250 μL of chemotaxis buffer at 5×10^6 cells/mL. The chamber was incubated for 4 h at 37 °C with 5% CO_2, and then the membrane was removed, washed, fixed, and stained with a Diff-Quik kit. Cell chemotaxis was quantified microscopically by counting cells in five high-power fields (× 40). The relative chemotactic index represented the mean number of cells migrating in response to ligand stimulation as compared to that without ligand stimulation.

Cdc42 Activity Assay

PBD (p21 binding domain)-based assays of CDC42 were performed as described by Benard et al. (13). Briefly, CXCR2 expressing HEK293 cells were stimulated with 50 ng/mL CXCL1 for the indicated time, and cells were immediately lysed by sonication in RIPA buffer containing cocktail protease inhibitor. Four hundred micrograms of protein of each whole cell extract was incubated with purified GST–PBD (GST-conjugated p21 binding domain) beads for 30 min at 4 °C. The bound GTP–Cdc42 and total level of Cdc42 were detected by Western blotting using a cdc42 polyclonal antibody (SC-87) (Santa Cruz Biotechnology).

Intracellular Ca^{2+} Mobilization

Chemokine-induced intracellular Ca^{2+} mobilization was measured as described by Wang et al. (39). Briefly, subconfluent CXCR2-expressing HEK293 cells transfected with vector, dominant negative PAK1, dominant negative cdc42, or dominant negative ERK1/2 were plated on glass-bottom microwells and grown overnight. Prior to the experiment, the cells were incubated in serum-free media for 3–4 h. The cells were then rinsed with wash buffer (10 mM Hepes, pH 7.4; 140 mM NaCl; 5mM KCl; 1 mM $MgCl_2$; and 0.55 mM glucose) and loaded with 1 μM Fluo-3 AM for 30 min at room temperature. After a wash with wash buffer, 1 mL of wash buffer containing 1mM $CaCl_2$ was added to the cells. The microwell was then placed on a Zeiss Axiovert 135 confocal microscope, and the cells were stimulated with CXCL1 (100 ng/mL) at room temperature. The emitted fluorescence at a wavelength of 488 nm was

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recorded. All images from the scanning were processed to analyze the change of relative fluorescence intensity at the single-cell level using the NIH Image program. The relative fluorescence intensity of each sample in the figures represents the mean of the relative fluorescence intensity of six randomly chosen fields (10 cells were counted in each field).

RESULTS

CXCL1 Induces PAK1 Activation

To determine whether CXCL1 induces PAK1 activation through activation of CXCR2, PAK1 kinase assays were performed to evaluate endogenous PAK1 kinase activity in the CXCR2-expressing HEK293 cells stimulated with CXCL1 for the indicated times. The results of these assays showed that CXCL1 stimulation of CXCR2-expressing HEK293 cells with CXCL1 increased the ability of PAK1 to phosphorylate myelin basic protein (MBP), which is a substrate of PAK1 (Figure 1A, top panel). The PAK1 activation started at 5 min, reached the maximum at 30 min, and was almost back to the basal level at 120 min. The expression level of PAK1 in the samples from the various time points was equivalent (Figure 1A, lower panel). In contrast, CXCL1 failed to induce PAK1 activation in parental HEK293 cells (data not shown). These data demonstrate that CXCL1 induces PAK1 activation through CXCR2.

PAK1 Mediates CXCL1-Induced Chemotaxis

Ligand-stimulated CXCR2-mediated chemotaxis is a direct and effective functional test to access the chemokine receptor signal transduction. Because PAK1 activation is involved in the regulation of cytoskeletal organization, it was of interest to determine whether PAK1 activation was required for CXCL1-induced chemotaxis. A dominant negative PAK1 (pCMV5M/PAK1 232 K/A) (38) was transfected into HEK293 cells stably expressing CXCR2 to determine whether loss of PAK1 activation could abolish the CXCR2-mediated chemotaxis in a modified Boyden chamber assay. This dominant negative form of PAK1 (232 K/A) has only a catalytic domain of PAK1 (amino acids 232–544) containing a point mutation that renders it inactive (K298A). Because it lacks the N-terminal regulatory domain, it cannot bind to Rac1 or Cdc42 (38). We observed a CXCL1 concentration-dependent chemotactic response in the control cells (CXCR2-expressing HEK293 cells transfected with empty expression vector of PAK1) with a peak migration occurring at a concentration of ~25 ng/mL CXCL1. Chemotaxis was inhibited at higher concentrations of CXCL1 (Figure 1B, empty bar), as reported earlier (36). In contrast, the expression of dominant negative PAK1 (232 K/A) resulted in a marked attenuation of CXCR2-mediated chemotaxis (Figure 1B, solid bar). In addition, the expression of a dominant negative PAK1 (R298), which lacks only kinase activity but can still bind Rac and cdc42, also blocked CXCL1-induced chemotaxis (data not shown). Because CXCL1 failed to induce a chemotactic response in the parental HEK293 cells (data not shown), these data demonstrate that PAK1 is required for CXCL1-stimulated CXCR2-mediated chemotaxis.

PAK1 Is a Downstream Target of Cdc42

Recent studies showed that PDK1 and Akt mediators activate PAK1 independent of activation of cdc42 and Rac (40,41). Because activation of another chemoattractant receptor, the fMLP receptor, activates cdc42 (13), we examined whether CXCL1 activation of CXCR2 would also enhance cdc42 activation. Cdc42 activation assays were performed to evaluate endogenous cdc42 activity in the CXCR2-expressing HEK293 cells stimulated with 50 ng/mL of CXCL1 for the indicated times. The stimulation of CXCL1 increased the amount of endogenous GTP-bound cdc42 (active form of cdc42) (Figure 2A, upper panel). The levels of total cdc42 (GTP-cdc42 + GDP-cdc42) from the different samples were equivalent (Figure 2A, lower panel). The profile of cdc42 activation is consistent with that of PAK1 activation. To determine whether PAK1 is a substrate of cdc42 in CXCR2-expressing HEK293 cells, we tested whether

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the inhibition of cdc42 activation by expression of the dominant negative cdc42 would block CXCL1-induced PAK1 activation. Figure 2B shows that the dominant negative cdc42 inhibited CXCL1-induced PAK1 activation. This experiment demonstrates that CXCL1-induced PAK1 activation is dependent on cdc42 activation. To further test whether cdc42 is involved in CXCL1-induced PAK1-mediated chemotaxis, modified Boyden chamber assays were performed. Figure 2C shows that a CXCL1 concentration-dependent chemotactic response was observed in the CXCR2-expressing HEK293 cells transfected with the empty vector control (Figure 2C, white bar), but not in the same cells transfected with the dominant negative cdc42 expression plasmid (Figure 2C, black bar). This experiment demonstrates that cdc42 is required for CXCL1-induced chemotaxis. Taken together, these experiments demonstrate that a cdc42–PAK1 cascade is involved in CXCL1-induced chemotaxis mediated through CXCR2.

ERK Is Not a Downstream Target of PAK1

Previous studies demonstrated that CXCL1 activated ERK1/2 in CXCR2 stably expressing HEK293 cells, but not in the parental HEK293 cells (36). Because PAK was shown to facilitate ERK kinase activation by phosphorylating MEK (42), we examined whether ERK is a downstream target of PAK1 in response to CXCL1. Expression of dominant negative PAK1 (232 K/A) in the CXCR2-expressing HEK293 cells did not block CXCL1-induced ERK activation (Figure 3A). The data demonstrate that in CXCR2-expressing HEK293 cells, ERKs are not downstream targets of CXCL1-induced PAK1. However, we could not exclude the possibility that ERK activation is involved in chemotaxis from these data. Therefore, to evaluate whether ERK activation is involved in CXCL1-induced chemotaxis, we examined the effects of expression of dominant negative ERK on CXCR2-mediated chemotaxis. The expression of dominant negative ERK failed to block CXCL1-induced chemotaxis, as compared to the vector control (Figure 3B). These data demonstrate that ERK activation is not required for CXCL1-stimulated CXCR2-mediated chemotaxis in HEK 293 cells.

CXCL1 Triggers Two Independent Signal Pathways To Activate PAK1 and ERK1/2, Respectively

To further determine whether CXCL1-induced PAK1 is independent of the MEK1–ERK kinase pathway, the MEK1/2 inhibitor, PD98059, was used to inhibit CXCL1-induced ERK activation. PD98059 is an effective and specific inhibitor of ERK-mediated signaling (43). Figure 4A confirms that 25 μM PD98059 abrogated the CXCL1-induced ERK activation. However, inhibition of the MEK–ERK pathway with 25 μM PD98059 had essentially no effect on CXCL1-induced PAK1 activation (Figure 4B). Similarly, PD98059 (10–50 μM) did not block CXCL1-induced chemotaxis (Figure 4C). This result is consistent with the results found with the expression of dominant negative ERK. Taken together, these data demonstrate that CXCL1-induced PAK1 activation is independent of the MEK-ERK cascade.

Cdc42–PAK1 and ERK1/2 Are Not Required for CXCL1-Induced Intracellular Ca^{2+} Mobilization

CXCL1 induces intracellular Ca^{2+} mobilization through CXCR2 in CXCR2-expressing HEK293 cells (36). Because the CXCL1 also induces cdc42-PAK1 and ERK1/2 activation, we examined whether PAK1, ERK1/2, or cdc42 is involved in the CXCL1-induced intracellular Ca^{2+} mobilization. We performed calcium mobilization assays using fluo-3 Am loaded HEK293 cells stably expressing CXCR2. Cells were stimulated with CXCL1, and free intracellular calcium localization was examined and quantified by confocal microscopy as described under Experimental Procedures (39). The results are presented in Figure 5. The expression of either dominant negative PAK1, ERK1/2, or cdc42 did not block CXCL1-induced intracellular Ca^{2+} mobilization, as compared to the vector control. These experiments

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demonstrate that the cdc42–PAK1 cascade and ERK are not involved in CXCL1-induced intracellular Ca^{2+} mobilization.

RBL-2H3 Cells

To test whether the biological functions of PAK1 in HEK293 cells can be observed in RBL-2H3 cells, we examined whether PAK1 activation is required for CXCL1-induced chemotaxis in RBL-2H3 cells. The CXCR2-expressing RBL stable clone cells were stimulated with CXCL1 for the indicated times. As shown in Figure 6A, CXCL1 also can increase PAK1 kinase activity in RBL-2H3 cells. For chemotaxis assays, the RBL-2H3 cells were transiently transfected with CXCR2 receptor and either dominant negative PAK1 (232 K/A) or empty vector control for PAK1. Figure 6B shows that the expression of dominant negative PAK1 (232 K/A) inhibited CXCL1-induced chemotaxis (sold bars). In addition, the expression of another dominant negative PAK1 (R298) also blocked CXCL1-induced chemotaxis (data not shown). Because CXCL1 failed to induce a PAK1 activation and a chemotactic response in the parental RBL-2H3 cells, these results demonstrated that PAK1 is required for CXCL1-stimulated CXCR2-mediated chemotaxis.

DISCUSSION

Ligand-bound receptors activate G proteins by catalyzing the exchange of GDP bound to the α subunit with GTP, leading to dissociation of α-GTP from the $\beta\gamma$ subunit. Several major intracellular signaling pathways are regulated by both α and $\beta\gamma$ subunits. These include the cAMP/PKA pathway, the MAP kinase pathway, and the phosphatidylinositol/calcium pathway. CXCL8 activation of the PI3-kinase pathway is required for human neutrophil migration. The overall mechanism(s) responsible for the CXCL1 activation of the PI3-kinase pathway is (are) likely to be the same for CXCL8 activation. PI3-kinase can regulate PAK activation through Rac/cdc42 (44). The activation of cdc42 in response to CXCL1 in CXCR2-expressing HEK293 cells is more delayed, peaking at 5–10 min, compared to the activation of cdc42 in response to fMLP in human neutrophils, where the peak activation occurs at 0.5–1 min. The time course for Rac activation in response to CXCL1 is similar to the cdc42 in CXCR2-expressing HEK293 cells (data not shown). These differences in time course of Rac and cdc42 activation might be due to (1) the differences between classic chemoattractants versus CXC chemokines, (2) fMLP receptor versus CXCR2 receptor; and/or (3) cell type differences.

To date, four PAKs have been cloned, PAK1–4 (45–48). PAK1, 2, and 4 participate in the regulation of cytoskeletal organization (15–19,45,47,48). PAK2 is involved in the regulation of apoptosis (45,48,49). It has been reported that PAK1 is required for endothelial and fibroblast cell motility induced by an immobilized fibronectin (50,51). Here, we demonstrate that PAK1 is required for chemokine gradient-directed cell movement (chemotaxis) by using dominant negative PAK1. The expression of a dominant negative PAK1 (R299), which is defective only in kinase activity, blocked CXCL-induced chemotaxis (data not shown). However, this mutant may be inhibiting chemotaxis by sequestering cdc42 because it can still bind to Rac and cdc42. Dr. Melanie Cobb's group developed a novel dominant negative PAK1 mutant (232 K/A), which lacks kinase activity and fails to bind Rac and cdc42. So this PAK1 mutant (232 K/A) blocks only endogenous PAK1 activity but does not sequester the endogenous cdc42 and Rac and inhibit their interactions with other effectors (38). In CXCR2-expressing HEK293 cells, this PAK1 mutant (232 K/A) also blocked the endogenous PAK1 activation induced by CXCL1 (data not shown). We used this dominant negative PAK1 (232 K/A) to test whether PAK1 activation is required for a chemokine gradient directional cell movement. Our data demonstrated that PAK1 is required for CXCL1-induced chemotaxis in both HEK293 and RBL-2H3 cells.

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PAKs have been shown to regulate the MAP kinases ERK, JNK, and/or p38 in response to stimuli from cytokines, chemoattractants, and various stresses in certain types of the cell (41, 45). In CXCR2-expressing HEK293 cells, ERK is not a downstream target of PAK1. Recently, published data indicated that PAKs phosphorylate key signaling components such as paxillin (52), myosin light chain kinase (19), and LIM kinase (18), all of which are involved in regulation of the cytoskeletal organization. We have not, however, determined the exact downstream targets for PAK in CXCR2-expressing HEK293 cells. Future studies will address these unsolved issues.

In general, G-protein coupled receptors activate ERK1/2 via a G$\beta\gamma$ subunit complex. The signals for ERK1/2 activation are independent of receptor-mediated effects on phosphatidylinositol hydrolysis, calcium flux, or inhibition of adenyl cyclase (53,54). Our earlier data showed that CXCL1 activates the Ras–MEKK cascade, which is an upstream signal transduction pathway for MEK–ERK activation (7). Here, we show that ERK1/2 are not downstream targets of PAK1. However, it has been reported that ERK activation down-regulates p38 MAP kinase activity (55). It is possible that the ERKs may be indirectly involved in CXCL1-induced chemotaxis by altering downstream signaling of PAK1. Our data demonstrate that ERK activation is not involved in CXCL1-induced chemotaxis in CXCR2-expressing HEK293 cells.

For the first time, we demonstrate here that the cdc42–PAK1 cascade is required for CXCL1-induced chemotaxis in the CXCR2-expressing HEK293 and RBL cells. The activation of cdc42–PAK1 by CXCL1 is insensitive to inhibition of MEK1/2–ERK. ERK activation is also not required for CXCL1-induced chemotaxis. Moreover, CXCL1-induced intracellular Ca^{2+} mobilization is independent of both the cdc42–PAK1 and MEK–ERK cascades. This conclusion is consistent with the previous observation that CXC-chemokine-induced calcium mobilization is mediated by a phospholipase C-β, protein kinase C, and the IP3 cascade (8). Taken together, our findings further define the signal transduction pathways for diverse biologic functions of CXCL1. Advances in the relationship between ligand biologic function and signal transduction pathways should lead to development of specific inhibitors, which can be useful for pharmacological targets.

Acknowledgements

We also are indebted to Dr. Gary Bokoch for providing GST-PBD/hPCR construct, Dr. Melanie Cobb for providing the mutant PAK1 (232 K/A) construct, and Xuejie Wang for assistance with calcium mobilization assays.

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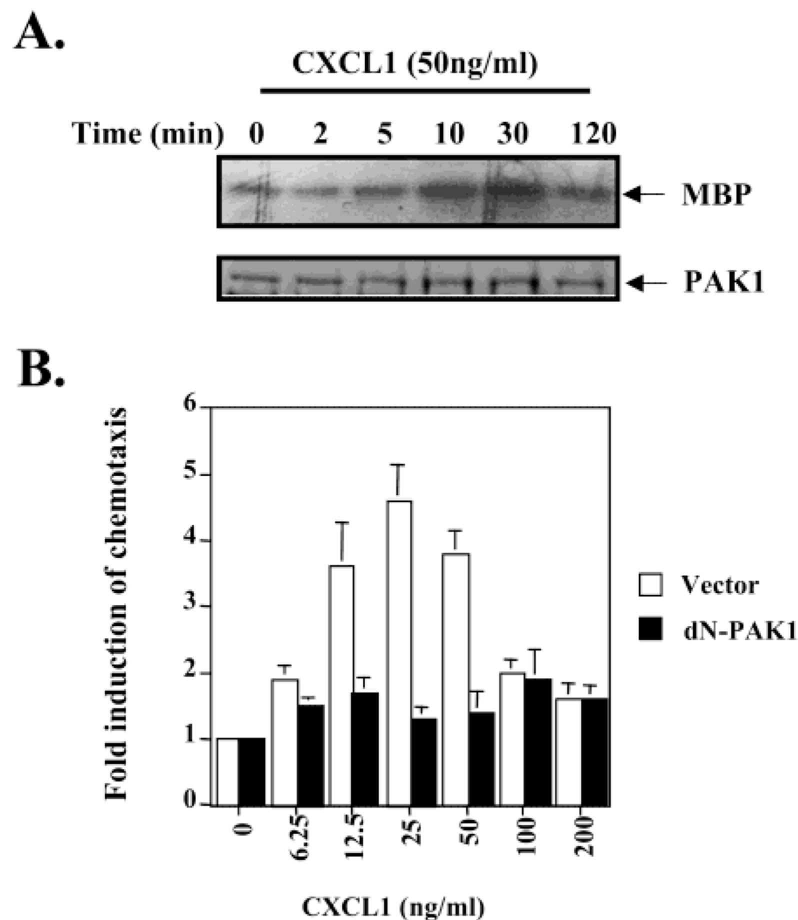

Figure 1.

CXCL1 induces PAK1 activity, which is required for chemotaxis in CXCR2-expressing HEK293 cells: (A) PAK1 kinase activity. CXCR2-expressing HEK293 cells were either untreated or treated with 50 ng/mL CXCL1 for the indicated times after serum starvation for 14 h. Endogenous PAK1 was immunoprecipitated by PAK1 antibody from whole cell extracts. PAK1 activity was determined by an immunocomplex kinase assay using MBP as a substrate as described under Experimental Procedures. Phosphorylated proteins were resolved on a 10% SDS–PAGE reducing gel and transferred to a nitrocellulose membrane. The phosphorylated MBP bands were visualized by autoradiography (upper panel). The blot was probed with PAK1 antibody to monitor equal loading of PAK1 (lower panel). This figure is representative of three different experiments with similar results. (B) Effect of the dominant negative PAK1 on CXCL1-stimulated CXCR2-mediated chemotaxis in CXCR2-expressing HEK293 cells. CXCR2-expressing HEK293 cells were transiently transfected with either the empty expression vector (white bars) or dominant negative PAK1 plasmid (black bars). Two days after transfection, cells were compared for chemotactic response to CXCL1 stimulation as described under Experimental Procedures. Values represent the means ± SE of three independent experiments. The data were analyzed using Student's paired *t* test ($p < 0.05$).

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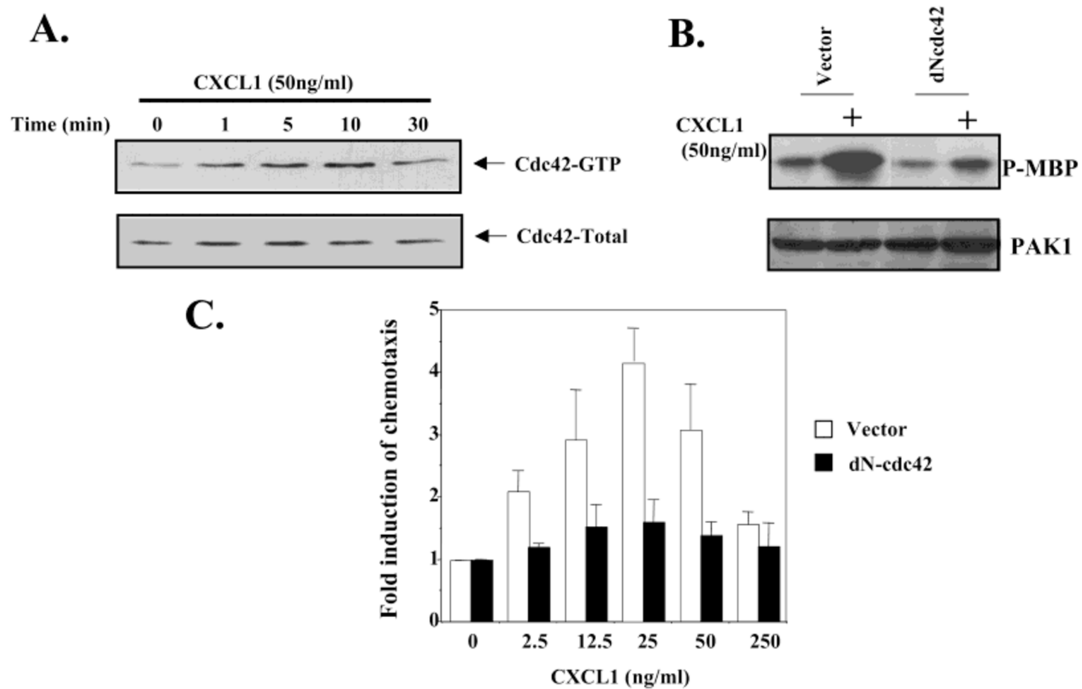

Figure 2.
CXCL1 induces cdc42 activation, which is required for chemotaxis: (A) Cdc42 activity. CXCR2-expressing HEK293 cells were either untreated or treated with 50 ng/mLCXCL1 for the indicated times after serum starvation for 14 h. Endogenous GTP-bound cdc42 was precipitated from whole cell extracts by GST-PBD and immunoblotted with cdc42 antibody. The lower panel represents the total cdc42 expression level from whole cell extracts as the loading control. This figure is representative of three different experiments with similar results. (B) Effect of dominant negative cdc42 on the CXCL1-induced PAK1 activation. CXCR2-expressing HEK293 cells were transfected with either vector or dominant negative cdc42 plasmid as described in Figure 1B. After 2 days, cells were either untreated or treated with 50 ng/mL CXCL1 for 10 min. The PAK1 activation was detected by in vitro PAK1 kinase assays as described in Figure 1A. The lower panel was reprobed with PAK1 antibody to monitor equal loading of PAK1. These figures are representative of three different experiments with similar results. (C) Effect of the dominant negative cdc42 on CXCL1-stimulated CXCR2-mediated chemotaxis in CXCR2-expressing HEK293 cells. CXCR2-expressing HEK293 cells were transiently transfected with either the empty expression vector (white bars) or dominant negative cdc42 plasmid (black bars). Two days after transfection, cells were compared for chemotactic response to CXCL1 stimulation as described under Experimental Procedures. Values represent the means ± SE of three independent experiments. The data were analyzed using Student's paired *t* test ($p < 0.05$).

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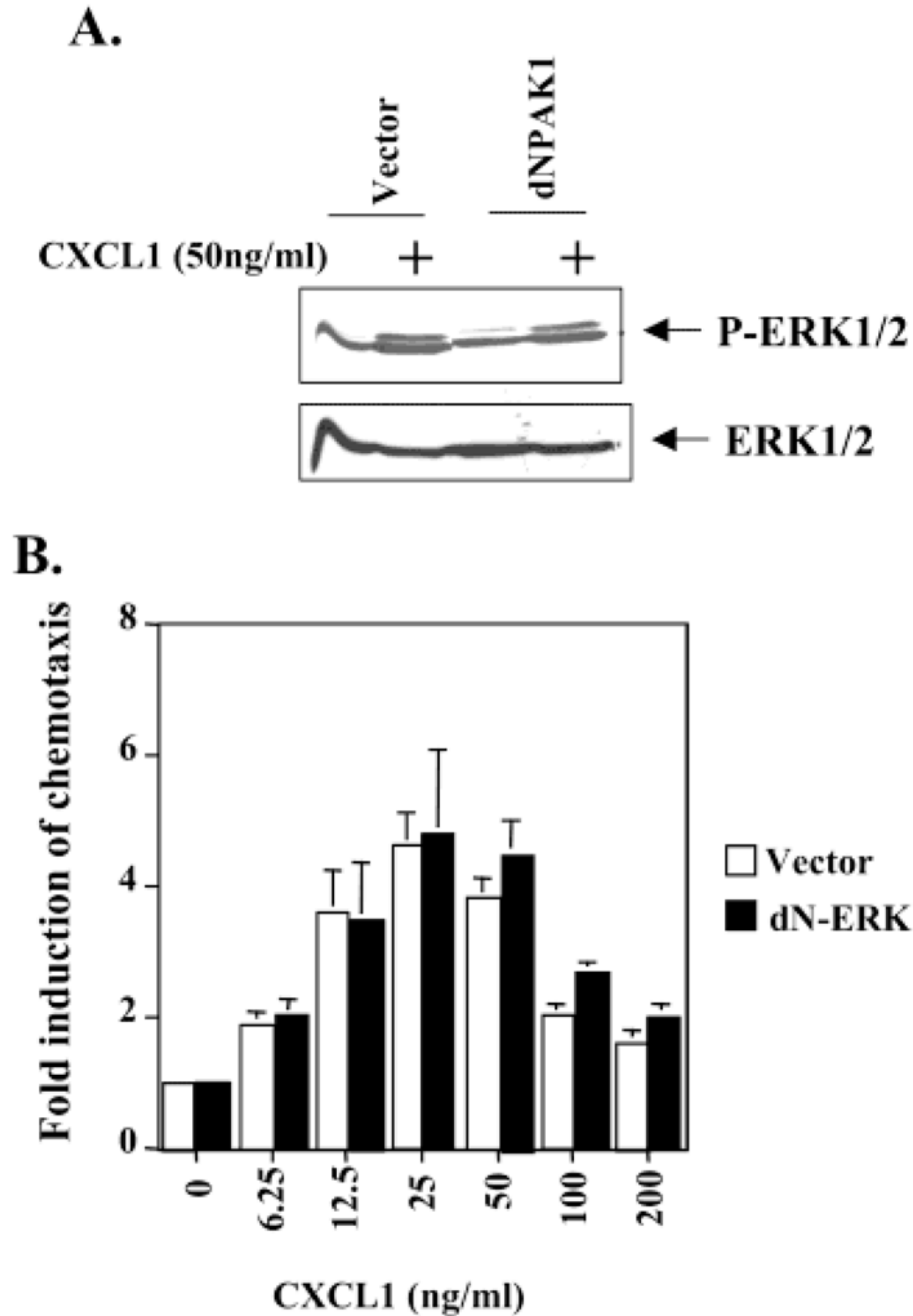

Figure 3.

ERK activation is not required for CXCL1-induced chemotaxis: (A) ERKs are not downstream targets of PAK1. CXCR2-expressing HEK293 cells were transfected with either vector or dominant negative PAK1 plasmid as described in Figure 1B. After 2 days, cells were either untreated or treated with 50 ng/mL CXCL1 for 10 min. The ERK1/2 activation was detected by Western blot as described in Figure 2A. The blot was reprobed with ERK antibody to monitor equal loading of ERK1/2 (lower right panel). These figures are representative of three different experiments. (B) Effect of the dominant negative ERK on CXCL1-stimulated CXCR2-mediated chemotaxis in HEK293 cells. CXCR2-expressing HEK293 cells were transiently transfected with either the empty expression vector (white bars) or dominant

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negative ERK plasmid (black bars). Two days after transfection, cells were compared for chemotactic response to CXCL1 stimulation as described under Experimental Procedures. Values represent the mean ± SE of three independent experiments performed in duplicate. The data were analyzed using Student's paired *t* test ($p < 0.05$).

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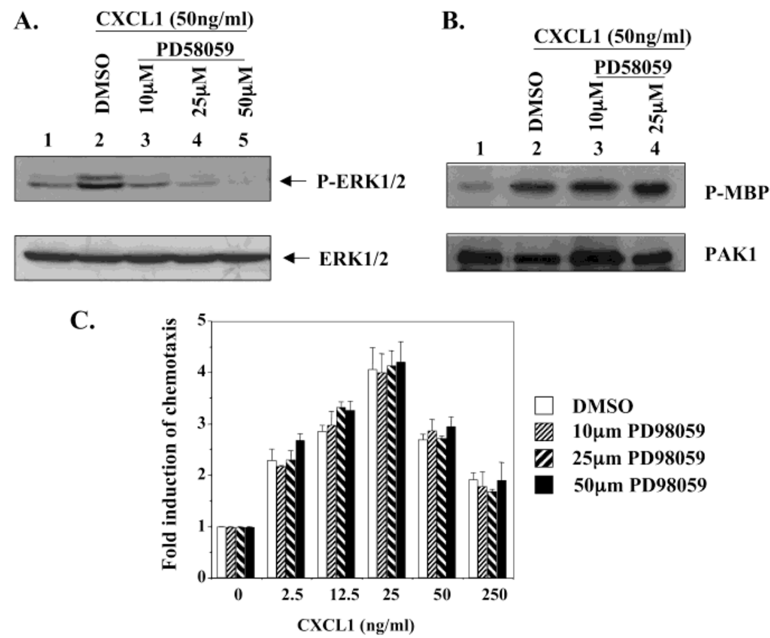

Figure 4.

MEK inhibitor fails to block PAK1 activation and chemotaxis: (A) MEK inhibitor, PD98059, blocks CXCL1-induced ERK activation. The CXCR2 stably expressing HEK293 cells were treated with DMSO or different concentrations of PD98059 overnight before stimulation with CXCL1. ERK activation assays were performed as described in Figure 3A. (B) PD98059 failed to inhibit CXCL1-induced PAK1 activation. CXCR2 stably expressing HEK293 cells were treated with DMSO or PD98059 as described above. PAK1 kinase assays were performed as described in Figure 1. (C) PD98059 did not inhibit CXCL1-induced chemotaxis. CXCR2-expressing HEK293 were treated with DMSO or PD98059 as described above. Chemotaxis assays were performed as described in Figure 1B. Values represent the means ± SE of three independent experiments. The data were analyzed using Student's paired *t* test ($p < 0.05$).

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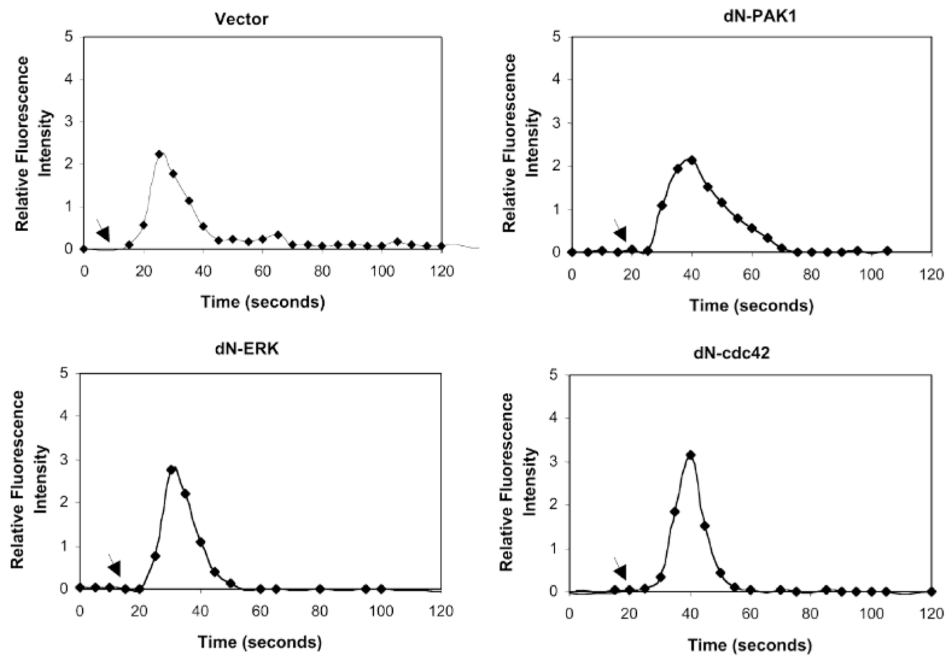

Figure 5.

CXCL1-induced Ca^{2+} mobilization does not require cdc42–PAK1 and ERK. Intracellular Ca^{2+} mobilization was monitored by confocal microscopy as described under Experimental Procedures. The curve represents the mean of the relative fluorescence intensity of six randomly chosen fields (10 cells were counted in each field). Arrows indicate the time point when the ligand was added to cells.

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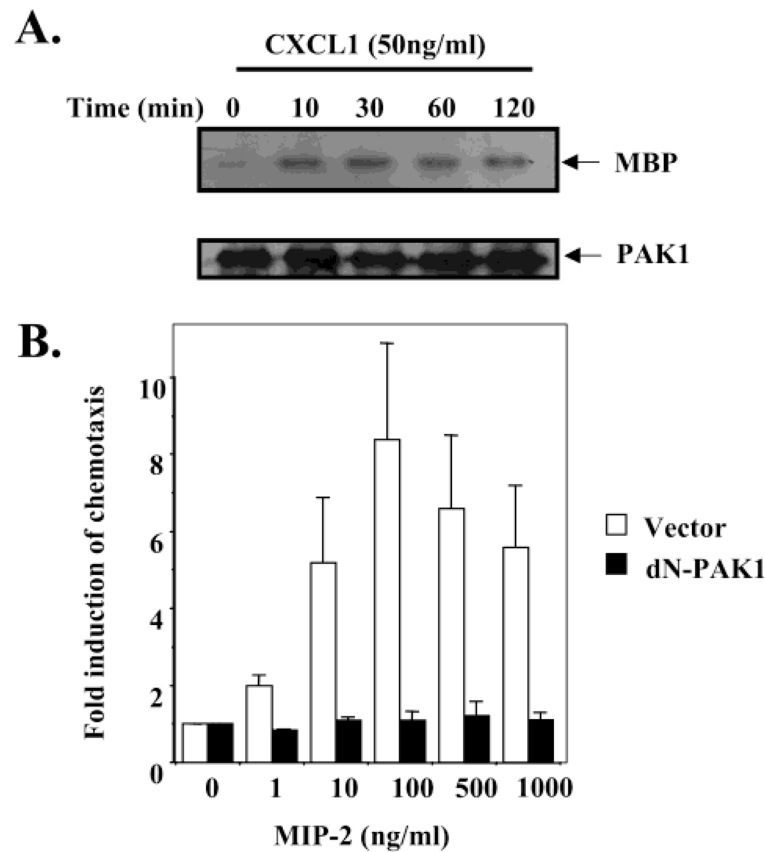

Figure 6.

PAK1 is required for CXCL1-induced chemotaxis in RBL-2H3 cells: (A) PAK1 kinase activity. The PAK1 activation in CXCR2-expressing RBL-2H3 cells was detected by in vitro PAK1 kinase assays as described in Figure 1A. The lower panel was reprobed with PAK1 antibody to monitor equal loading of PAK1. These figures are representative of three different experiments with similar results. (B) Effect of dominant negative PAK1 (232 K/A) on CXCL1-induced PAK1 activation in RBL-2H3 cells. RBL-2H3 cells were transiently cotransfected with CXCR2 and either vector or dominant negative PAK1 plasmid, and chemotaxis assays were performed as described in Figure 1B. Values represent the means ± SE of three independent experiments. The data were analyzed using Student's paired *t* test ($p < 0.05$).