

# Regulation of CXCR4-Mediated Nuclear Translocation of Extracellular Signal-Related Kinases 1 and 2

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## ABSTRACT

Activation of the chemokine receptor CXCR4 by its agonist stromal cell-derived factor 1 (SDF-1) has been associated with cell migration and proliferation in many cell types, but the intracellular signaling cascades are incompletely defined. Here we show that CXCR4-dependent extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation was mediated through the Ras/Raf pathway, as demonstrated with a dominant-negative Ras mutant and pharmacological inhibitors. The Src inhibitor 4-amino-5-methylphenyl-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) and the Rho-kinase (ROCK) inhibitor *N*-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride (Y27632) also attenuated SDF-1-induced ERK1/2 phosphorylation. Involvement of Src could furthermore be demonstrated by Src phosphorylation and by the shortened ERK1/2 phosphorylation in SYF cells, which are Src/Yes/Fyn-deficient compared with Src-reconstituted Src<sup>++</sup> cells. Mem-

brane translocation of RhoA could be detected similarly. A large portion of the SDF-1-mediated ERK phosphorylation was detected in the nucleus, as shown by Western blotting and confocal microscopy, and resulted in the phosphorylation of the transcription factor Elk. It is interesting that the nuclear accumulation of ERK1/2 and Elk phosphorylation was completely blocked by dominant-negative Rho, Y27632, PP1, and latrunculin B, indicating that the Rho/ROCK pathway, Src kinase, and the actin cytoskeleton were required in this process. In accordance, neither nuclear ERK phosphorylation nor Elk phosphorylation were observed in SYF cells stimulated with SDF-1 but were reconstituted in Src<sup>++</sup> cells. In summary, these results demonstrate that Src, Rho/ROCK, and an intact cytoskeleton contribute to overall ERK1/2 activation in SDF-1-stimulated cells and are indispensable for nuclear translocation of ERK1/2 and activation of transcription factors.

The chemokine stromal cell-derived factor 1 (SDF-1/CXCL12) and its receptor, CXCR4, play an important role in various cellular responses, including chemotaxis, angiogenesis, and cell proliferation (Rossi and Zlotnik, 2000). CXCR4 also serves as a coreceptor of T-tropic human immunodeficiency virus-1 entry into mononuclear cells. Numerous signaling molecules, including G<sub>i</sub>, Ras, PI-3 kinase, nuclear factor- $\kappa$ B, and ERK, have been implicated in CXCR4 signaling in different cell types. It has been established that

CXCR4 signaling pathways are activated in a range of cancer cells, in which their activation has been associated with proliferation and metastatic dissemination (Muller et al., 2001; Kijima et al., 2002; Schrader et al., 2002; Taichman et al., 2002; Zhou et al., 2002).

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation is mediated by numerous seven transmembrane spanning receptors (7TM-Rs), but the activation mechanism varies for different receptors (Luttrell et al., 2001). In a classic module, ligand stimulation of 7TM-Rs activates Ras on the plasma membrane, which in turn activates c-Raf, MEK1/2, and ultimately ERK1/2. Other signaling regulators have been implicated in control of ERK1/2 activation, including Src,  $\beta$ -arrestin, protein kinase C, PI-3 kinase, and Rho (Luttrell et al., 2001). These different mechanisms contribute

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**ABBREVIATIONS:** SDF-1, stromal cell-derived factor 1; 7TM-R, seven transmembrane receptor; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinases 1 and 2; ROCK, Rho-kinase; DN-Rho, dominant-negative Rho; GFP, green fluorescent protein; HMEC, human microvascular endothelial cells; PI-3, phosphatidylinositol-3; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinases 1 and 2; PMSF, phenylmethanesulfonyl fluoride; RFP, red fluorescent protein; PP1, 4-amino-5-methylphenyl-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; DAPI, 4,6-diamidino-2-phenylindole; PTX, pertussis toxin; FAK, focal adhesion kinase; latB, latrunculin B; Egr-1, early growth response factor-1; Y27632, *N*-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride; FTI-277, methyl[*N*-[2-phenyl-4-*N*(2*R*)-amino-3-mecaptopropylamino] benzoyl]-methionate; PD98059, 2'-amino-3'-methoxyflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; AG-1478, 4-(3'-chloroanilino)-6,7-dimethoxy-quinazoline.

to the fine-tuning of ERK1/2 activation in response to diverse stimuli. Duration, intensity, and subcellular compartmentalization of ERK1/2 play roles in numerous cellular activities, including cell proliferation. ERK1/2-mediated cell proliferation is dependent on the activation of transcription factors after nuclear translocation of ERK1/2.

ERK1/2 does not possess a nuclear localization signal, and the nuclear entry of ERK1/2 is not an obligatory consequence after activation (Osawa et al., 2004; Whitehurst et al., 2004a). ERK1/2 accumulation in the nucleus depends on the nature of the stimulus, but it is independent of the intensity of ERK1/2 activation (Whitehurst et al., 2004b). Although the underlying mechanism of ERK1/2 translocation into the nucleus remains unclear, the subcellular distribution of ERK1/2 can be regulated by a number of signaling molecules (e.g.,  $\beta$ -arrestin, PEA-15, and MEK). MEK is reported to interact with ERK, maintaining ERK in the cytoplasm (Fukuda et al., 1997). Upon growth factor stimulation, ERK undergoes phosphorylation and dissociates from MEK. Likewise,  $\beta$ -arrestin has been shown to mediate ERK activation by acting as a scaffolding protein. It, too, retains ERK1/2 in the cytoplasm instead of promoting ERK translocation to the nucleus (DeFea et al., 2000). This cytoplasmic ERK activation has different functions from nuclear activation, because it has been implicated in Ras-induced senescence (Gaumont-Leclerc et al., 2004), but it can also be antiapoptotic (Ajenjo et al., 2004). The nuclear export factor PEA-15 has been reported to regulate the actions of the ERK MAPK cascade. PEA-15, which contains a nuclear export sequence, binds ERK1/2 and sequesters it in the cytoplasm, blocking ERK-dependent transcription and proliferation (Formstecher et al., 2001). In contrast, growth factor receptor-bound protein 2-associated binder-1 has a putative nuclear localization signal, and its interaction with ERK1/2 plays a role in ERK1/2 translocation and Egr-1 accumulation in the nucleus (Osawa et al., 2004). Recent reports also suggest that RhoA/ROCK activation is involved in regulation of ERK1/2 translocation to the nucleus, but the details of this pathway have not been elucidated (Kawamura et al., 2003; Liu et al., 2004).

After entry into the nucleus, phosphorylated ERK1/2 can activate several transcription factors, including Elk and early growth response factor-1 (Egr-1). Elk is responsible for transcriptional activation of the immediate early gene *c-fos*. Thus, Elk activation reflects the entry of ERK1/2 from the cytoplasm to the nucleus. A second transcription factor, Egr-1, which is up-regulated by nuclear ERK activation, plays a regulatory role in the expression of growth factors, cytokines, and adhesion molecules. It is responsible for the increased expression of growth factors, such as platelet-derived growth factor, cytokines, and adhesion molecules.

CXCR4-mediated ERK1/2 activation has been reported in several cell types, and CXCR4 signaling has been associated with cell proliferation in normal and cancer cells. However, the intracellular signaling pathways, which are elicited in ERK1/2 activation-mediated cell proliferation during these processes, are poorly understood. Activation of CXCR4 in human megakaryoblasts led to the phosphorylation of the nuclear transcription factor Elk (Majka et al., 2000). Likewise, Egr-1 expression was increased by SDF-1 in arterial endothelial cells, thus enhancing vascular endothelial growth factor-mediated cell proliferation (Neuhaus et al., 2003). Both of these observations imply nuclear translocation

of ERK, but this has not been experimentally confirmed. Here, we report that CXCR4 activation induced nuclear translocation of ERK1/2 and phosphorylation of the transcription factor Elk. This pathway is dependent on Src kinase, Rho/ROCK activation, and an intact actin cytoskeleton, a pathway that has been described previously for integrin-dependent nuclear translocation of ERK1/2 (Aplin et al., 2001), but not for 7TM-Rs.

## Materials and Methods

**Materials.** CXCR4 cDNA was polymerase chain reaction-amplified and inserted into pcDNA3.1 (Invitrogen, Carlsbad, CA). The plasmid encoding CXCR4-GFP and the expression vector encoding red fluorescence protein (RFP)-tagged ERK2 have been described previously (Zhao et al., 2004). Dominant-negative Rho (DN-Rho) was generously provided by Dr. Gary Bokoch (The Scripps Research Institute, La Jolla, CA). FTI-277, Raf-1 kinase inhibitor, PD98059, Y27632, LY294002, and latrunculin B were purchased from Calbiochem (San Diego, CA). PP1 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA), and pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA). Anti-c-Raf, anti-RhoA, anti-actin, and anti-lamin B antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-Elk (Ser383), anti-Elk, anti-phospho-ERK1/2, anti-ERK1/2, and anti-phospho-Src antibodies were from Cell Signaling Technology Inc. (Beverly, MA); and anti-Src monoclonal antibody was from Upstate Biotechnology (Lake Placid, NY). Protein A beads were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and [ $\gamma$ -<sup>32</sup>P]ATP was from PerkinElmer Life and Analytical Sciences (Boston, MA). SDF-1 was expressed in *Escherichia coli* and purified as described previously (Zhao et al., 2004).

**Cell Culture and Transfection.** Human embryonic kidney (HEK) epithelial 293 cells; HMECs, a human microvascular endothelial cell line; SYF, Src/Yes/Fyn-deficient mouse embryonic fibroblasts; and Src<sup>++</sup> cells (Klinghoffer et al., 1999), their Src-reconstituted counterpart, were obtained from American Type Culture Collection (Manassas, VA). HEK293, SYF, and Src<sup>++</sup> cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. HMECs were cultured in endothelial basal medium (Cambrex Bio Science Walkersville, Walkersville, MD). HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen). Stable cell lines were selected with 800 mg/ml G418. All cells were serum-starved overnight before SDF-1 stimulation.

**Western Blotting.** Monolayer cells were stimulated as described in the figure legends, then lysed with modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 10% glycerol, 1% Nonidet P-40, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM EDTA, 2 mM PMSF, 2 mg/ml leupeptin, 2 mg/ml aprotinin, 2 mM sodium pyrophosphate, 2 mM sodium vanadate, and 10 mM NaF), and clarified by centrifugation. Immunoprecipitation assays were performed as described previously (Zhao et al., 2004). The clarified cell lysates or immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 3% dry milk in Tris-buffered saline/Tween 20, and exposed to specific primary antibodies as described for each experiment. Antibody binding was detected using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies and enhanced chemiluminescence (GE Healthcare). Phosphoblots were reprobed with a second antibody (e.g., anti-ERK1/2 antibody) to ensure equal loading. UN-SCAN-IT gel digitizing software (Silk Scientific Inc., Orem, UT) was used to quantify results.

**Preparation of Membrane Fraction.** To isolate membrane fractions, cells were stimulated for the indicated times and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 2 mM EDTA, 1 mM DTT, 2 mM PMSF, 2 mg/ml leupeptin, and 2 mg/ml aprotinin) by sonication. Nuclei and cell debris were removed by centrifugation at 500g for 10 min. The supernatants

were subjected to ultracentrifugation at 100,000g for 40 min, and the pellets were washed once. Supernatant fractions (cytoplasm) and pellet fractions (membranes) were separated by SDS-PAGE.

**Isolation of Cell Nuclear Fraction.** Cells were vortexed in buffer A (10 mM HEPES, pH 8.0, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 2 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) containing 0.5% Nonidet P-40 and then microfuged at 12,000g for 2 min. The pellets were washed with buffer A, then resuspended in buffer B (20 mM HEPES, pH 8.0, 250 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 2 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin), and incubated on ice for 15 min followed by another 2-min centrifugation at 4°C. The supernatants (nuclear fraction) were transferred to clean tubes containing 4× Laemmli sample buffer, boiled, and separated by SDS-PAGE. To exclude cross-contamination between nuclear and cytoplasmic fractions, fractionated samples were probed with a nuclear marker (lamin B) and with actin, which is excluded from the nucleus.

**Raf Kinase Assay.** Experiments were carried out as described previously (Zhao et al., 2004). In brief, washed c-Raf immunoprecipitates were suspended in 40 μl of kinase buffer (30 mM HEPES, pH 7.4, 10 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM β-glycerophosphate, and 50 μM ATP) containing 15 mCi [ $\gamma$ -<sup>32</sup>P] ATP and 0.4 μg of purified kinase-inactive MEK1 (Upstate Biotechnology) and then incubated at 30°C for 30 min. Kinase reactions were stopped by adding Laemmli sample buffer and boiling, followed by separation by SDS-PAGE electrophoresis, gel transfer, and autoradiography. Membranes were probed with anti-c-Raf antibody to ensure equal loading.

**Actin Polymerization.** Serum-starved cells on collagen-coated coverslips were pretreated with various inhibitors before stimulation with SDF-1 and fixed with 4% paraformaldehyde in PBS for 20 min followed by permeabilization with 0.2% Triton X-100 for 5 min. Cells were then stained with Alexa 488 phalloidin (Invitrogen) according to the manufacturer's instructions. Images were taken on an Olympus FV1000 confocal microscope (Melville, NY).

**Fluorescence Microscopy.** HEK293 cells expressing CXCR4-GFP were grown on collagen-coated glass coverslips. After SDF-1 stimulation, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with methanol for 10 min at -20°C. After blocking with 3% BSA for 2 h at room temperature, cells were incubated with anti-phospho-ERK1/2 for 1 h, followed by staining with Alexa Fluor 568 anti-mouse IgG<sub>1</sub> monoclonal antibody (Invitrogen) in 1% BSA for 1 h at room temperature. Cells were then stained with DAPI (Sigma-Aldrich, St. Louis, MO) for 10 min and washed twice with PBS before mounting with AntiFade (Invitrogen). Images were taken on an Olympus FV1000 confocal microscope.

To examine the translocation of total ERK to the nucleus, HEK293 cells transiently cotransfected with different combinations of GFP and RFP plasmids were treated with SDF-1 for 5 min in the presence or absence of inhibitors, and confocal images were captured as above.

## Results

**CXCR4-Mediated ERK1/2 Phosphorylation Depends on the Ras/Raf Pathway.** To determine ERK1/2 signaling pathways used by CXCR4, HMEC cells, which express the CXCR4 constitutively, or HEK293, cells stably transfected with CXCR4 were stimulated with SDF-1 for the indicated times. In both cell types, SDF-1 elicited a rapid and robust ERK1/2 phosphorylation, reaching a maximum at 5 min (Fig. 1, A and B). Pretreatment with the farnesyl transferase inhibitor FTI-277 which blocks Ras, or expression of a dominant-negative mutant of Ras in CXCR4-293 cells attenuated this response (Fig. 1C). As expected, a Raf-1 inhibitor and the MEK inhibitor PD98059 significantly reduced ERK1/2 phosphorylation (Fig. 1D). In addition, c-Raf activity could also be

detected directly in a peptide kinase assay in SDF-1-stimulated CXCR4-293 cells (Fig. 1E). These results indicate that CXCR4-elicited ERK1/2 activation is mediated by the Ras/Raf pathway.

**Involvement of Multiple Signal Regulators in CXCR4-Mediated ERK1/2 Activation.** To explore the signaling pathways involved in CXCR4-mediated ERK1/2 activation, inhibitor screening was performed. Pertussis toxin (PTX) abolished ERK1/2 phosphorylation (Fig. 2A), indicating that G<sub>i</sub> was essential in this process. Because Src has been shown to play a role in MAPK activation of some 7TM-Rs (Luttrell et al., 1999; Klinger et al., 2002), the effect of the Src inhibitor PP1 was tested next. Inhibition of Src family kinases largely blocked SDF-1-induced ERK1/2 activation in a dose-dependent fashion (Fig. 2B). To exclude that this effect was caused by nonspecific effects of PP1, ERK1/2 phosphorylation was also assessed in SYF cells, a mouse embryonic fibroblast cell line derived from Src/Yes/Fyn triple knockout embryos that expresses CXCR4 constitutively. In these cells, SDF-1-dependent ERK1/2 phosphorylation was more short-lived than in Src<sup>++</sup> cells, which are SYF cells reconstituted with Src (Fig. 2C). Because Src phosphorylation is a complex process, phosphorylation of a single amino acid usually shows only a subtle response. Autophosphorylation of Tyr416 in the activating loop of the kinase domain on Src is known to be required for Src kinase activation. In Fig. 2D, SDF-1 stimulation caused a visible increase in phosphorylation of Src on Tyr416 in both CXCR4-293 and HMECs. Because focal adhesion kinase (FAK) is a kinase that is downstream of Src activation, tyrosine phosphorylation of FAK was also determined and was found to increase after the addition of SDF-1 (Fig. 2D).

The PI-3-kinase inhibitor LY294002 showed only a minor effect on ERK1/2 activation in HEK293 cells, and the EGF receptor blocker AG1478 failed to inhibit the ERK1/2 phosphorylation (results not shown), even though it has been reported that the EGF receptor can be transactivated by SDF-1 (Porcile et al., 2004).

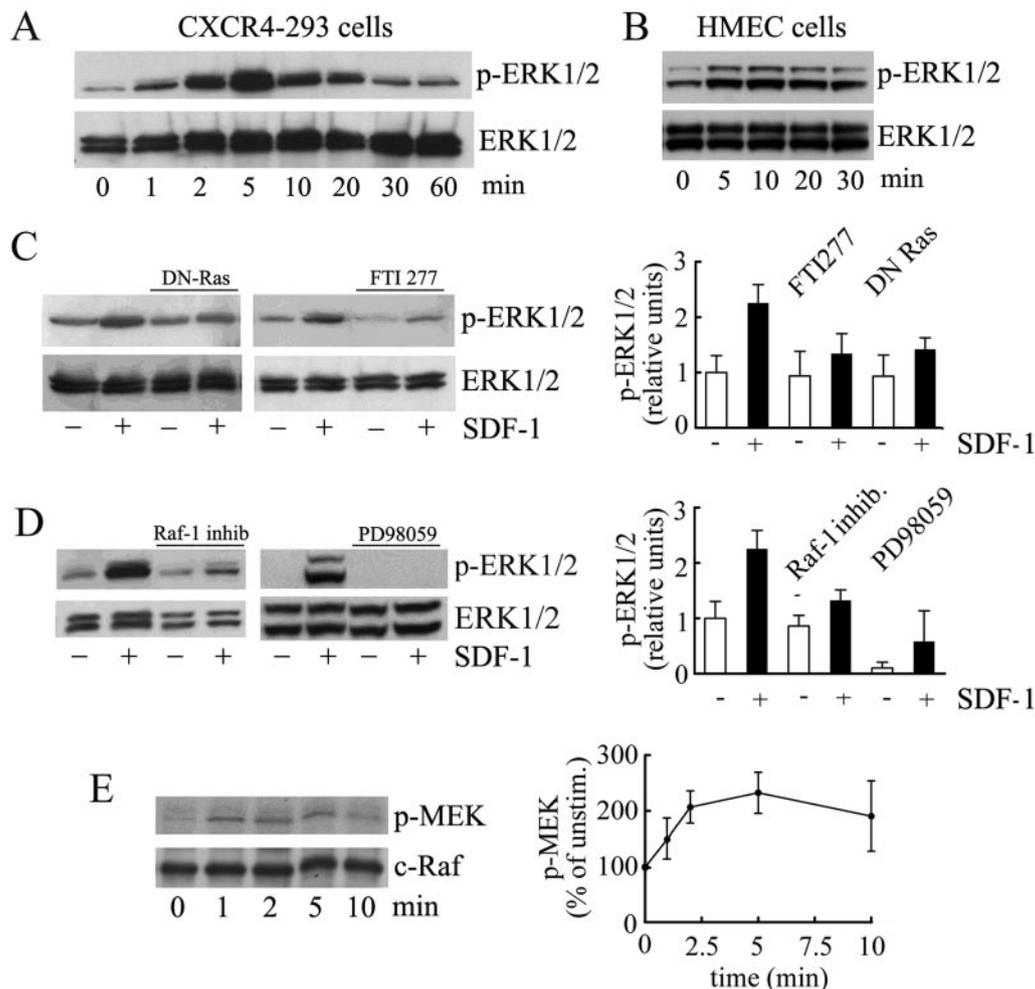
It is more surprising that the ROCK inhibitor Y27632 was found to attenuate SDF-1-induced ERK1/2 phosphorylation in CXCR4-HEK293 cells (Fig. 2E). DN-Rho also showed partial inhibition of SDF-1-induced ERK1/2 activation (Fig. 2E). These results strongly suggested that the Rho/ROCK pathway participated in the regulation of CXCR4-mediated ERK1/2 activation. Because inhibition of the Rho cascade attenuated ERK1/2 activation, RhoA translocation to the membrane, an indication of RhoA activation (Hirakawa et al., 2004), was determined next to confirm RhoA involvement. In isolated plasma membrane fractions from CXCR4-293 cells stimulated with SDF-1, increasing RhoA was detected by Western blotting (Fig. 2E), suggesting that RhoA was activated by SDF-1. Because the RhoA/ROCK pathway is usually linked with actin cytoskeletal changes, we next determined whether an intact actin cytoskeleton is required for SDF-1-induced ERK1/2 phosphorylation. Cytoskeletal disruption by pretreatment of cells with latrunculin B (latB) largely reduced SDF-1-induced ERK1/2 phosphorylation (Fig. 2F), suggesting an essential role of the intact actin cytoskeleton in ERK1/2 activation. To exclude a general toxic effect of latB, the effect of latB on ERK1/2 phosphorylation was also determined in HEK293 cells stimulated with EGF. As shown in Fig. 2F, EGF-induced ERK1/2 phosphorylation

was barely affected in the presence of latB. This result indicated that HEK293 cells retained the ability to respond with ERK activation in the presence of latB and ruled out a non-specific effect of latB on the MAPK cascade.

**SDF-1 Induced Nuclear Translocation of ERK1/2.** Because CXCR4 can mediate cell proliferation through ERK1/2 activation, we next determined whether ERK translocation to the nucleus could be detected. To exclude cross-contamination of the nuclear and cytoplasmic fractions, both fractions were immunoblotted with actin as a cytoplasmic marker and lamin-B as a nuclear marker. As shown in Fig. 3A, carryover of proteins was negligible. In CXCR4-293 cells, SDF-1 induced rapid and strong ERK1/2 phosphorylation in the nucleus, as shown by Western blot in Fig. 3B. A sizeable portion of nuclear ERK1/2 was phosphorylated, as evident from the additional bands of slightly higher molecular weight representing the phosphorylated forms of the protein, which are visible in the nuclear fractions in blots of total ERK1/2

(for instance, in Fig. 3B). PP1, an inhibitor of Src family kinases, largely inhibited ERK1/2 translocation (Fig. 3C), suggesting that Src family kinases were required in this process. As before, the inhibitor study was complemented by genetic evidence. In SYF cells depleted of Src family kinases, no nuclear accumulation of ERK1/2 could be observed after stimulation with SDF-1 (Fig. 3D). This behavior was reversed in the Src<sup>++</sup> cell line, in which Src expression is restored (Fig. 3D), and this activation could be overcome by PP1 (Fig. 3D). These results indicated that Src expression was sufficient to mediate this effect but do not rule out an overlapping or redundant role of Yes and Fyn in this process. Moreover, latrunculin B abolished the nuclear import of ERK1/2 (Fig. 3E), suggesting that an intact actin cytoskeleton was required in this process. Likewise, both DN-Rho and Y27632 largely blocked ERK1/2 phosphorylation in the nucleus (Fig. 3E).

To confirm the biochemical analysis, nuclear translocation



**Fig. 1.** CXCR4-mediated ERK1/2 activation through the Ras/Raf pathway. Cells were serum-starved overnight, then stimulated with 500 ng/ml SDF-1 for the indicated times, whole-cell lysates were separated by SDS-PAGE, and ERK1/2 activation was determined by immunoblotting with anti-phospho-ERK1/2 antibody (top) followed by total ERK1/2 detection (bottom) to ensure equal loading. **A**, time course of SDF-1-induced ERK1/2 phosphorylation in HEK293 cells expressing CXCR4. **B**, time course of ERK1/2 phosphorylation in HMECs stimulated with SDF-1. **C**, effect of Ras inhibition on ERK1/2 phosphorylation. CXCR4-293 cells were cotransfected with DN-Ras or were pretreated with FTI-277, a farnesyltransferase inhibitor, and stimulated with SDF-1 for 5 min. For **D**, on the left is a Western blot, and on the right, quantification (mean  $\pm$  S.D.,  $n = 3$ ); the effect of inhibition of Raf and MEK is shown. HEK293 cells expressing CXCR4 were preincubated with Raf-1 inhibitor or the MEK inhibitor PD98059 and stimulated with SDF-1 for 5 min. Again, the left shows a representative Western blot, and the right shows the quantification (mean  $\pm$  S.D.,  $n = 3$ ). **E**, MEK1 phosphorylation assay. CXCR4-expressing HEK293 cells were stimulated with SDF-1 for the indicated times. Raf was immunoprecipitated, and a kinase assay was performed as described under *Materials and Methods*. Left, top row: autoradiography of <sup>32</sup>P-phosphorylated *p*-MEK; bottom, Western blot loading control. Right, quantification of the results of three experiments (mean  $\pm$  S.D.).

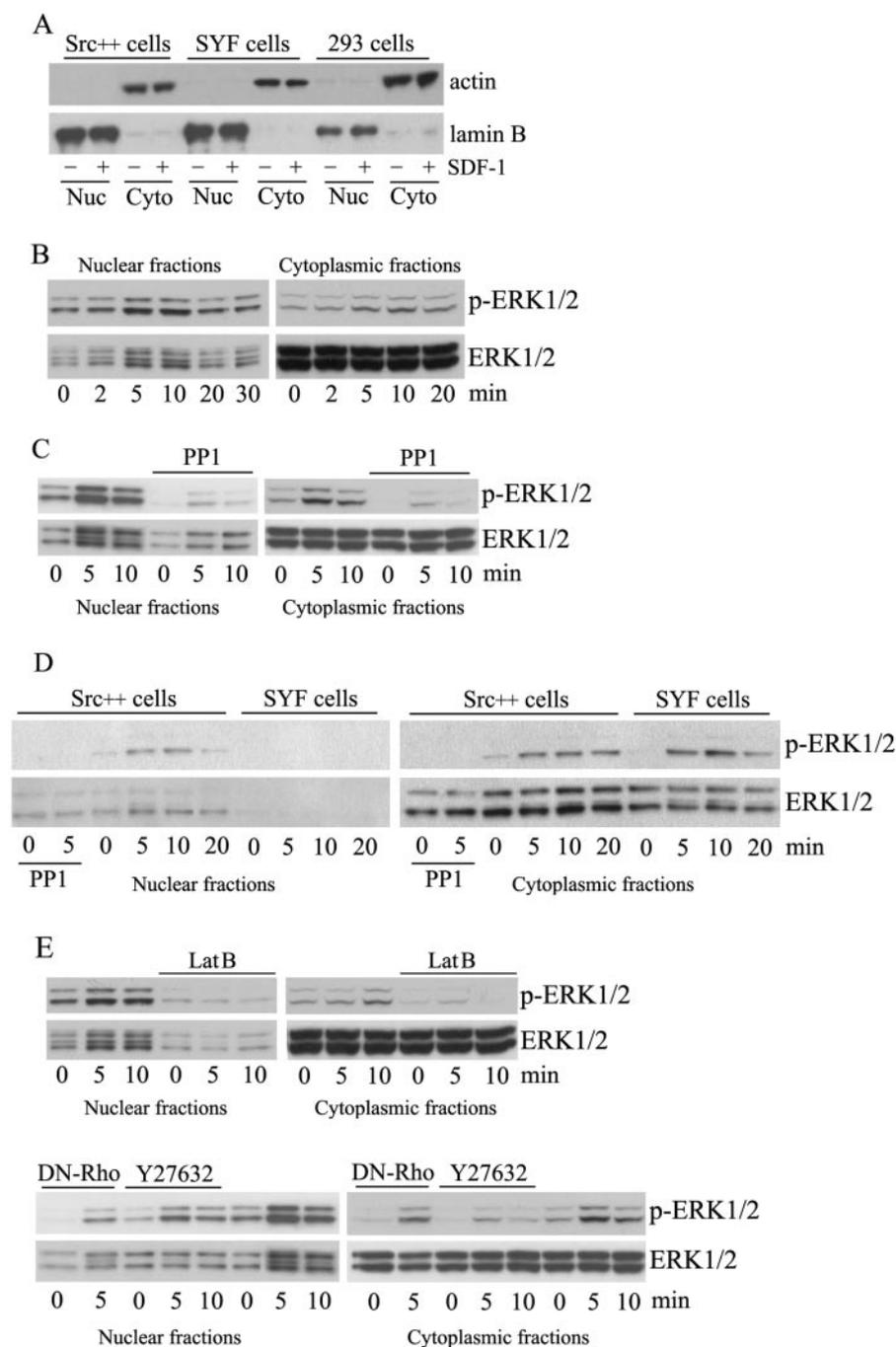


phospho-ERK1/2 could be detected in the nucleus counterstained with DAPI (Fig. 4B). Again, this was prevented in the presence of the inhibitors described above (Fig. 4B). From these results, it seems that SDF-1-induced ERK1/2 translocation from the cytoplasm to the nucleus involves RhoA/ROCK, Src, and the actin cytoskeleton.

**Stress Fiber Formation.** Disruption of the actin cytoskeleton prevented SDF-1-induced nuclear translocation of ERK1/2, as indicated by the effect of latrunculin B (Fig. 4). RhoA/ROCK involvement also suggested that the actin skeleton was required for CXCR4-mediated nuclear translocation of ERK1/2. Although SDF-1 induced actin polymerization in HEK293 cells (Fig. 5A), these cells do not show prominent stress-fiber formation in general. Therefore, HMECs, which

respond with prominent stress-fiber formation, were used for inhibitor studies. In these cells, SDF-1-induced actin polymerization was easily detectable and lasted for at least 20 min (Fig. 5B). Inhibition studies showed that PP1, Y27632, DN-Rho, and latrunculin B blocked SDF-1-induced stress-fiber formation (Fig. 5C). These results were consistent with the above conclusion and suggested that Src and the RhoA/ROCK pathways were intimately linked in CXCR4 signaling, coordinating ERK1/2 activation and nuclear translocation.

**Nuclear Translocation of ERK1/2 Increased Phosphorylation of Transcription Factors.** Nuclear ERK1/2 activation has been shown to activate a number of transcription factors, including Elk and Egr-1. The transcription factor Elk is a direct target of ERK1/2 and is phosphorylated at



**Fig. 3.** SDF-1-induced nuclear translocation of ERK1/2. CXCR4-293 cells, SYF, and Src<sup>++</sup> cells were serum-starved overnight and stimulated with SDF-1 for various times, cell lysates were separated into nuclear and cytoplasmic fractions, and cells were immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. A, immunoblot of fractionated samples using anti-lamin B (a nuclear marker) and anti-actin (a cytoplasmic marker) antibodies to exclude cross-contamination between nuclear and cytoplasmic fractions. B, time course of SDF-1-induced nuclear translocation of ERK1/2 in CXCR4-293 cells. C, effect of the Src inhibitor PP1 on SDF-1-induced nuclear translocation of ERK1/2 in CXCR4-293 cells. D, SDF-1-induced nuclear translocation of ERK1/2 in SYF and Src<sup>++</sup> cells. E, effect of the actin-disrupting agent, latrunculin B, and of inhibition of Rho/ROCK with Y27632 or dominant-negative Rho on SDF-1-induced nuclear translocation of ERK1/2 in CXCR4-293 cells.

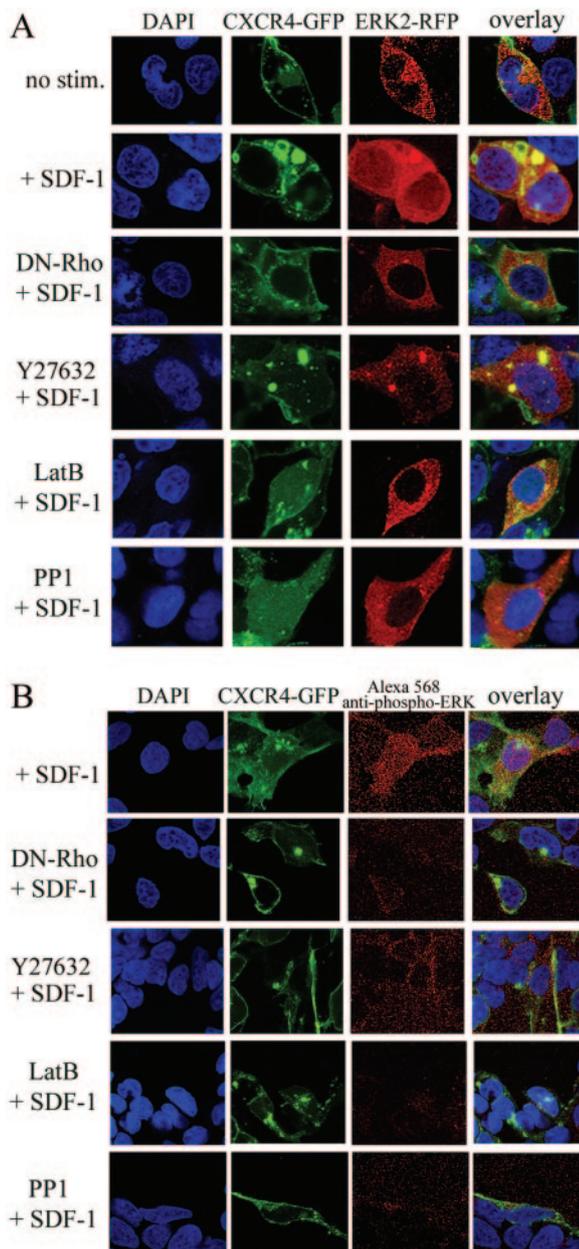
Ser382 when activated. To assess the consequence of CXCR4-mediated ERK1/2 entry into the nucleus, phosphorylation of Elk was determined in cell lysates from CXCR4-293 cells, SYF, and Src<sup>++</sup> cells stimulated with SDF-1 for various times by using anti-phospho-Elk antibody. In agreement with the above results that SDF-1 induced nuclear import of ERK1/2, Elk phosphorylation was observed as early as 5 min after stimulation, showing a time course similar to that of

ERK1/2 activation (Fig. 6A). Pretreatment of cells with the MEK inhibitor PD98059 abolished Elk phosphorylation, which was direct evidence that Elk phosphorylation was dependent on ERK1/2 activation. All inhibitors that had been shown to prevent nuclear translocation of ERK1/2 (PP1, Y27632, DN-Rho, and latB) completely blocked SDF-1-induced Elk phosphorylation (Fig. 6B). As expected, SDF-1-induced Elk phosphorylation could be detected in Src<sup>++</sup> cells but not in SYF cells (Fig. 6C), which was strong evidence that Src is required for CXCR4-mediated nuclear ERK1/2 and Elk activation.

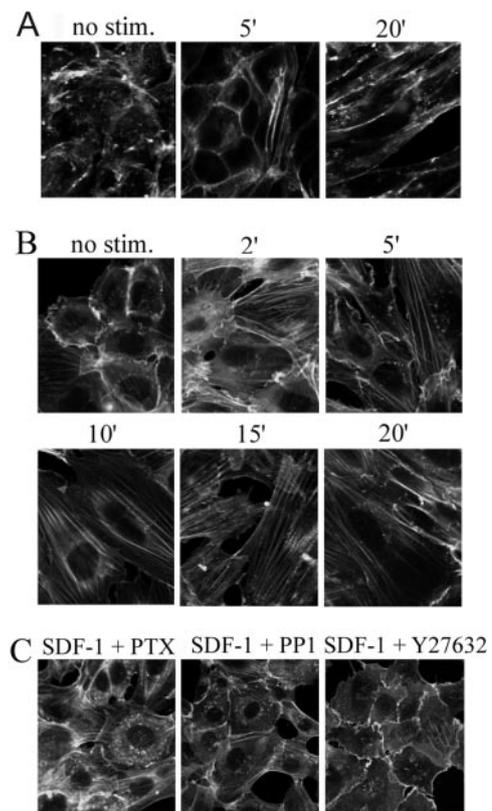
## Discussion

In this study, we found that SDF-1 can induce ERK1/2 nuclear translocation and Elk activation in an Src-, Rho/ROCK-, and F-actin-dependent manner. This explains the ability of CXCR4 to promote cell proliferation. We also observed higher levels of ERK phosphorylation in the nucleus than in the cytoplasm, suggesting that the nuclear ERK activation is a major target in CXCR4-mediated MAPK signaling.

In general, the mechanisms of nuclear translocation of ERK1/2 are incompletely understood. They are particularly intriguing, however, because of the opposite effects of nuclear phospho-ERK1/2, which promotes proliferation (Brunet et al., 1999), and cytoplasmic phospho-ERK1/2, which can in-



**Fig. 4.** Confocal fluorescence microscopy of ERK1/2 cellular distribution. A, HEK293 cells on coverslips were transiently transfected with a minimum amount of ERK2-RFP and CXCR4-GFP. Cells were pretreated with various inhibitors for 30 min before the addition of SDF-1 for 5 min. Nuclei were stained with DAPI, and ERK2-RFP entry into the nucleus was detected by confocal microscopy. B, HEK293 cells expressing CXCR4-GFP cultured on coverslips were pretreated with various inhibitors as indicated, and SDF-1-induced nuclear ERK1/2 phosphorylation was detected by immunofluorescence confocal microscopy as described under *Materials and Methods*. Cellular distribution of CXCR4-GFP, Alexa 568-stained phospho-ERK1/2, and DAPI-stained nuclei are shown in single-channel and overlay images.



**Fig. 5.** SDF-1 induced stress-fiber formation in CXCR4-293 and HMEC cells. Cells on coverslips were stimulated with 500 ng/ml SDF-1 as indicated, fixed, and stained with Alexa 488 phalloidin. Actin polymerization was viewed by confocal microscopy. A, cytoskeletal reorganization in HEK293 cells expressing CXCR4. B, SDF-1 induced actin polymerization in HMEC cells. C, HMEC cells were incubated with various inhibitors for 30 min before stimulation with SDF-1 for 10 min. Actin polymerization was detected as described above.

duce senescence (Gaumont-Leclerc et al., 2004) and differentiation (Smith et al., 2004). This ERK activation in the nucleus seems of particular significance in the case of CXCR4, because this receptor is expressed by a variety of metastatic cancer cell. Although internalization of CXCR4 depends on the presence of  $\beta$ -arrestin (Orsini et al., 1999), it is difficult to show complex formation between the CXCR4,  $\beta$ -arrestin, and the MAPK cascade (M. Zhao and I. Schraufstatter, unpublished results), as is possible for the  $\beta_2$ -adrenergic receptor (Luttrell et al., 1999) or CXCR2 (Zhao et al., 2004). Because  $\beta$ -arrestin complexes with the MAPK cascade retain ERK1/2 in the cytoplasm (DeFea et al., 2000), it is perhaps the transience or low affinity of these complexes in the case of CXCR4 that allows nuclear translocation of ERK1/2.

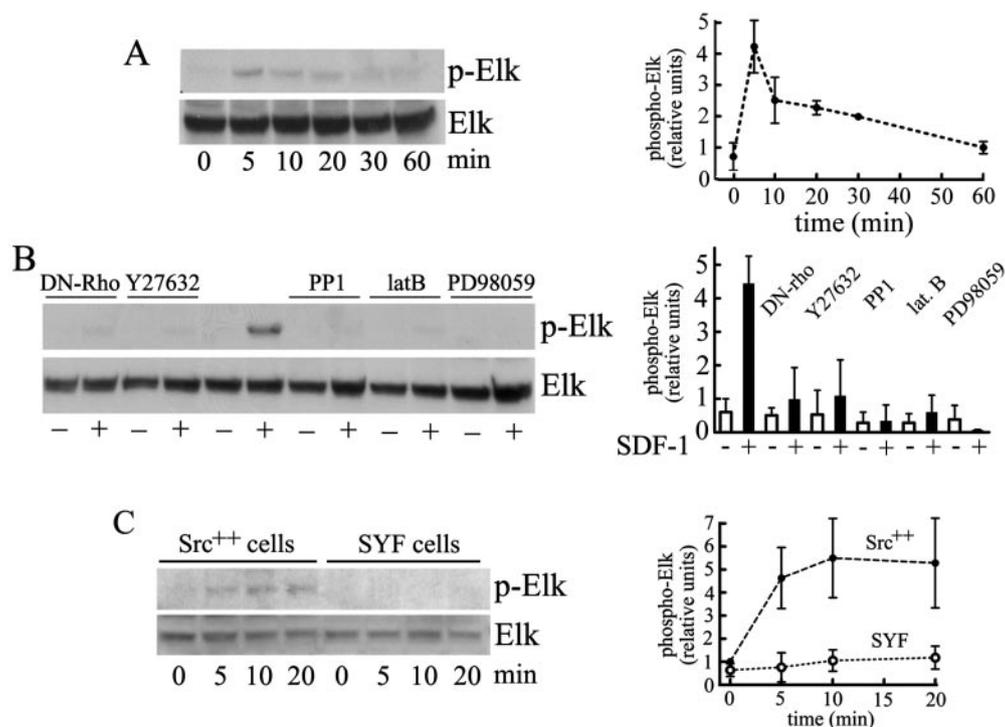
Although nuclear ERK phosphorylation is a common response for 7TM-Rs, we are only aware of a single report in which nuclear transport of ERK1/2 was shown to be caused by Rho/ROCK activation. In serotonin-stimulated smooth muscle cells, ROCK inhibition blocked nuclear ERK1/2 activation and Elk phosphorylation, but in contrast to our study, it showed no effect on overall ERK phosphorylation (Liu et al., 2004). It is possible that in the case of the serotonin-stimulated cells, a larger fraction of the ERK1/2 activation occurred in the cytoplasm, whereas in our study, the majority of ERK1/2 phosphorylation took place in the nucleus. It has also been shown by confocal microscopy that RhoA or Rac1 activation is involved in stretch-induced nuclear translocation of ERK1/2 (Kawamura et al., 2003). The Rho/ROCK pathway was also reported to regulate nuclear export signal through the nuclear membrane (Lee et al., 2004). It seems that Rho/ROCK can act as a switching molecule that manages the influx or efflux of signal molecules to the nucleus, thus regulating various cell activities.

The RhoA/ROCK pathway plays an important role in the regulation of the cytoskeleton, which in turn plays a role in cell migration, cell-cycle progression, apoptosis, and cancer

development. Although  $G\alpha_{12}$  and  $G\alpha_{13}$  are best known as the G-proteins that cause Rho/ROCK activation and actin stress-fiber formation, activation of these pathways in SDF-1-stimulated cells was downstream of  $G\alpha_i$ , as indicated by the inhibitory effect of pertussis toxin. The ROCK inhibitor Y27632 attenuated SDF-1-induced overall ERK activation but almost completely prevented the nuclear translocation of ERK1/2 and abrogated Elk phosphorylation. Whereas ROCK activation seems to be upstream of ERK phosphorylation, one may speculate that its effect is indirect. One possible explanation would be that actin polymerization was necessary for translocation of signaling molecules to the plasma membrane. It has been described that translocation of Src to the plasma membrane is mediated by the actin cytoskeleton under the control of Rho family proteins (Fincham et al., 1996). On the other hand, ROCK activity may promote nuclear translocation of ERK and/or of transcription factors such as myocardin-related transcription factors (called MRCF or MAL) (Miralles et al., 2003).

The Rho/ROCK pathway has been implicated in CXCR4-mediated lymphocyte organization, chemotaxis, calcium mobilization, and cancer cell invasion (Bug et al., 2002; Bartolome et al., 2004). In CXCR4-293 cells, the Rho/ROCK pathway and an intact actin cytoskeleton not only influenced nuclear translocation of ERK1/2, but also regulated the overall ERK1/2 activation. This regulation may rely on Raf-1 phosphorylation, because it has been reported that stretch-induced Raf-1 activation requires integrity of the actin cytoskeleton (Krepinsky et al., 2005).

The actin cytoskeleton has been implicated in numerous cellular activities, including chemotaxis and exocytosis. Disruption of the actin cytoskeleton with latrunculin B abolished CXCR4-mediated ERK1/2 activation, indicating a critical role of the intact cytoskeleton in CXCR4 signaling. Disruption of caveolae in neonatal rat cardiomyocytes abolished stretch-induced RhoA and Rac activation, impaired



**Fig. 6.** Phosphorylation of the transcription factor Elk. **A**, time course of Elk phosphorylation: HEK293 cells expressing CXCR4 were stimulated with SDF-1 for different times, whole-cell lysates were separated by SDS-PAGE, and Elk phosphorylation was detected by Western blotting (left). Right, quantification of these results (mean  $\pm$  S.D.,  $n = 3$ ). **B**, effect of inhibitors on Elk phosphorylation: cells were cotransfected with DN-Rho or were pretreated with various inhibitors for 30 min before the addition of SDF-1; Elk activation was evaluated by Western blotting. Left, a representative blot; right, quantification (mean  $\pm$  S.D.,  $n = 3$ ). **C**, time course of Elk phosphorylation in SYF and Src<sup>++</sup> cells: SYF and Src<sup>++</sup> cells were serum-starved overnight and then treated with SDF-1 for the indicated times; Elk phosphorylation was examined as described above. Left, Western blot; right, quantification (mean  $\pm$  S.D.,  $n = 3$ ).

actin polymerization and ERK1/2 translocation to nucleus, but had no effect on overall ERK activation (Kawamura et al., 2003). Although an intact actin cytoskeleton has been shown to be required for ERK1/2 translocation to the nucleus in this case, disruption of the cell cytoskeleton had no effect on ERK1/2 phosphorylation or nuclear translocation of ERK1/2 induced by activation of the serotonin receptor 1B/1D in smooth muscle cells (Liu et al., 2004). Finally cytoskeletal disruption can even have the opposite effect. In retinoic acid-treated cells, an intact cytoskeleton is required to restrict activated ERK1/2 to the cytoplasm, and actin-disrupting agents lead to increased nuclear ERK1/2 activation (Smith et al., 2004). The role of the cytoskeleton in nuclear translocation of ERK1/2 clearly needs further investigation. Actin polymerization is not a general requisite for nuclear translocation of ERK1/2, and inhibition of the actin response had no effect on EGF-mediated nuclear ERK activation (Fig. 2F).

We hypothesize that the intact cytoskeleton played a role in the initial translocation of signaling molecules, including translocating Src to the plasma membrane (Fincham et al., 1996). Involvement at this stage is also suggested by the observation that inhibitors which blocked nuclear translocation of ERK1/2 (PP1, Y27632, and latrunculin B) also inhibited internalization of SDF-1-stimulated CXCR4-GFP, as detected by confocal microscopy (M. Zhao and I. Schraufstatter, unpublished results), indicating that the Rho/ROCK pathway and the actin cytoskeleton were involved in the initial assembly of signaling molecules at the plasma membrane. This signaling cascade resembles that reported for integrin-dependent ERK1/2 activation (Fincham et al., 2000), a notion that is supported by the previously described integrin-activating effect of SDF-1 (Burger et al., 2003; Cardones et al., 2003). Integrin engagement has furthermore been shown to be involved in the nuclear translocation of ERK1/2 (Aplin et al., 2001). In this scenario, F-actin serves as a scaffold for efficient signaling.

Results from previous reports and those of ours indicate that SDF-1 stimulation leads to Src family kinase activation (Chernock et al., 2001; Ptasznik et al., 2002). This occurred upstream of ERK1/2 phosphorylation. Although PP1 is a relatively specific inhibitor, it inhibits all Src family kinases. Use of SYF and Src<sup>++</sup> cells, however, provided evidence that Src itself is sufficient for the ERK nuclear translocation and showed that this pathway is of physiological relevance in cells that express a low copy number of CXCR4 constitutively, underscoring that the nuclear translocation of ERK and Elk activation are not the consequence of overexpression of the receptor. There remained, however, a fraction of ERK phosphorylation that was not amenable to inhibition by Src family inhibitors/knockouts, which was retained in the cytoplasm. Because it was shown previously that dominant-negative arrestin can attenuate SDF-1-mediated ERK phosphorylation (Zhao et al., 2004), this fraction of phospho-ERK may have resulted from complex formation with  $\beta$ -arrestin, as has been shown for the PAR-2 (DeFea et al., 2000) and angiotensin type 1A receptors (Tohgo et al., 2002). As expected, FAK, another downstream signal of Src, was also phosphorylated after SDF-1 stimulation, as described previously for a human hematopoietic progenitor cell line (Wang et al., 2000). Because FAK can cause paxillin recruitment to the plasma membrane followed by activation of Rho family proteins

(Igishi et al., 1999), FRNK, a FAK inhibitor protein, was transfected together with CXCR4-GFP and RFP-ERK1/2. FRNK, however, did not seem to block nuclear translocation of RFP-ERK1/2 (results not shown), suggesting that FAK activation and ERK1/2 nuclear translocation were both downstream of Src activation but were independent of each other.

Nuclear ERK activation stimulates transcription factors such as Elk and Egr-1, which are known to regulate cell proliferation. In addition to the Elk phosphorylation described in this study, we also observed moderately increased Egr-1 expression in 293 cells upon stimulation of CXCR4 by using a luciferase activity assay, which was inhibited in the presence of Y27632 (results not shown). In agreement with the dependence of nuclear translocation of ERK1/2 on Src, Rho/ROCK, and polymerized actin, Elk phosphorylation was blocked by the same inhibitors. In contrast, activation of Elk after stimulation with lysophosphatidic acid depended on Rho but not on F-actin (Gineitis and Treisman, 2001).

In summary, we showed evidence that CXCR4 activation mediated nuclear translocation of ERK1/2 by using two different methods, Western blotting and confocal microscopy. It seems that the Src and RhoA/ROCK pathways not only regulated overall ERK1/2 activation mediated by CXCR4 but primarily controlled the nuclear translocation of ERK/2 and thereby influenced the activity of transcription factors. Moreover, these results suggest that cytoskeletal reorganization after agonist stimulation may play an important role in regulating the subcellular distribution of ERK1/2.

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