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Stromal Cell-Derived Factor 1α Activates LIM Kinase 1 and Induces Cofilin Phosphorylation for T-Cell Chemotaxis

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Stromal cell-derived factor 1 α (SDF-1α), the ligand for G-protein-coupled receptor CXCR4, is a chemotactic factor for T lymphocytes. LIM kinase 1 (LIMK1) phosphorylates cofilin, an actin-depolymerizing and -severing protein, at Ser-3 and regulates actin reorganization. We investigated the role of cofilin phosphorylation by LIMK1 in SDF-1α-induced chemotaxis of T lymphocytes. SDF-1α significantly induced the activation of LIMK1 in Jurkat human leukemic T cells and peripheral blood lymphocytes. SDF-1α also induced cofilin phosphorylation, actin reorganization, and activation of small GTPases, Rho, Rac, and Cdc42, in Jurkat cells. Pretreatment with pertussis toxin inhibited SDF-1α-induced LIMK1 activation, thus indicating that Gi protein is involved in LIMK1 activation. Expression of dominant negative Rac (DN-Rac), but not DN-Rho or DN-Cdc42, blocked SDF-1α-induced activation of LIMK1, which means that SDF-1α-induced LIMK1 activation is mediated by Rac but not by Rho or Cdc42. We used a cell-permeable peptide (S3 peptide) that contains the phosphorylation site (Ser-3) of cofilin to inhibit the cellular function of LIMK1. S3 peptide inhibited the kinase activity of LIMK1 in vitro. Treatment of Jurkat cells with S3 peptide inhibited the SDF-1α-induced cofilin phosphorylation, actin reorganization, and chemotactic response of Jurkat cells. These results suggest that the phosphorylation of cofilin by LIMK1 plays a critical role in the SDF-1α-induced chemotactic response of T lymphocytes.

Cell migration plays an essential role in a variety of physiological and pathological events, including wound healing, inflammation, immune responses, embryogenesis, organogenesis, angiogenesis, and tumor invasion and metastasis. Chemokines, a family of low-molecular-weight chemotactic proteins that have the potential to induce directional migration of cells, are grouped into four subfamilies, the CXC (α), CC (β), C (γ), and CX3C (δ) chemokines, based on the difference in the spacing of conserved cysteine residues (47). Stromal cell-derived factor 1α (SDF-1α), initially identified as a growth-stimulating factor for B-cell progenitors (34), is a member of the CXC chemokine subfamily that is expressed in a broad range of tissues and has multiple biological activities toward diverse cell types (47). SDF-1α has chemotactic activity toward several cell types, including T lymphocytes, monocytes, pre-B lymphocytes, dendritic cells, and hematopoietic cells (3, 10, 11, 16, 29, 49, 50, 56). The chemotactic activity of SDF-1α is mediated by a seven-transmembrane G protein-coupled receptor, CXCR4, which is also known as a coreceptor for T-cell-tropic human immunodeficiency virus strains (10). Targeted disruption of the SDF-1α gene or the CXCR4 gene in mice revealed that SDF-1α and CXCR4 have critical functions in the fetal development of the hematopoietic, cardiovascular, and cerebellar systems (33, 63). More recently, it has been shown that CXCR4 and SDF-1α are highly expressed in breast cancer cells and in organs representing the main sites of breast cancer metastasis, respectively, and that SDF-1α promotes metastasis by stimulating the migration of breast cancer cells (32). Therefore, SDF-1α and its receptor CXCR4 apparently play a role in determining the metastatic destination of breast cancer cells.

Reorganization of the actin cytoskeleton is central for cell migration (40) and is an early cellular response of chemotactic cells after chemokine stimulation (11, 49). The actin-binding protein cofilin plays a critical role in actin reorganization by depolymerizing and severing actin filaments (7, 13, 30). Cofilin, expressed ubiquitously in all eukaryotic cell types, is essential for cell motility and cell viability (1, 14, 22, 31, 48). The activity of cofilin is negatively regulated by a serine/threonine kinase, LIM kinase 1 (LIMK1), through phosphorylation at Ser-3 of cofilin (6, 58). We reported that cofilin phosphorylation by LIMK1 is a critical signaling event in semaphorin 3A-induced growth cone collapse of dorsal root ganglion neurons (4). Rho family small GTP-binding proteins, including Rho, Rac, and Cdc42, play a central role in regulating actin cytoskeletal reorganization through various downstream effector proteins (8, 24, 37, 43, 44). LIMK1 is activated through phosphorylation at Thr-508 by ROCK and PAK, which are downstream effectors of Rho and Rac/Cdc42, respectively (17, 28, 38). Therefore, both Rho-ROCK and Rac-PAK signaling pathways activate LIMK1, which in turn phosphorylates cofilin to regulate actin reorganization.

While understanding of the diverse biological functions of SDF-1α has progressed, little is known about the signaling mechanisms by which SDF-1α and its receptor CXCR4 induce effects on a variety of cells. Here we investigated the role of LIMK1 in SDF-1α-induced chemotaxis in T lymphocytes and provide evidence that SDF-1α induces LIMK1 activation that is mediated by Gi protein and Rac and that cofilin phosphor-

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ylation by LIMK1 is critical for SDF-1α-induced actin reorganization and chemotactic response of T lymphocytes.

MATERIALS AND METHODS

Materials. SDF-1α and pertussis toxin (PTX) were purchased from PeproTech (London, England) and Life Technologies (Gaithersburg, Md.), respectively. Mouse monoclonal antibodies to HA (12CA5) and Myc (9E10) eiptopes were obtained from Roche Diagnostics (Tokyo, Japan). Mouse monoclonal antibody to RhoA and rabbit polyclonal antibody to Cdc42 were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Mouse monoclonal antibody to Rac was purchased from Upstate Biotechnology (Lake Placid, N.Y.). Rabbit polyclonal antibody to LIMK1 (C-10) was generated as described previously (39). Rabbit polyclonal antibody to Ser-3-phosphorylated cofilin (P-cofilin) was prepared as described previously (54). Mouse monoclonal antibody to cofilin (MAB-22) was provided from T. Obinata (Chiba University) (2). Mouse monoclonal antibody to β-actin was purchased from Sigma (St. Louis, Mo.). Protein A-Sepharose and glutathione-Sepharose were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). The synthetic S3 and RV peptides were designed and synthesized as described previously (4).

Plasmids. Expression plasmids coding for HA- or Myc-tagged LIMK1 and Myc-tagged LIMK1 mutant (T508V) were constructed as described previously (38). Plasmids for HA-RhoN19, HA-RacN17, and HA-Cdc42N17 in pEF-BOS vector were provided by S. Kuroda and K. Kaibuchi (Nagoya University). Plasmids for βARK1ct in the pCMV vector were provided by H. Itoh (Tokyo University). The cDNA of the Rho-binding domain of rhotekin (amino acids 7 to 89) (RBD) was isolated from mouse brain cDNA by PCR and inserted into the pGEX2T vector. The pGEX2T-p21-binding domain of PAK1 (amino acids 67 to 150) (PBD) was a gift from H. Sumimoto (Kyushu University).

Cells and transfection. Jurkat human leukemia T cells, obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan), were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Human peripheral blood mononuclear cells were isolated by density gradient centrifugation, using Mono-Poly resolving medium (Dainippon Pharmaceutical, Osaka, Japan). Peripheral blood lymphocytes (PBL) were obtained by removing monocytes by plastic adherence for 1 h at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. For transient expression in Jurkat cells, about 10^7 cells were mixed with expression plasmids in 400 μl of electroporation medium (RPMI 1640 medium containing 20% fetal calf serum and 25 mM HEPES [pH 7.4]) and electroporated at 280 V and 975 μF, using a Gene Pulser II (Bio-Rad, Hercules, Calif.).

In vitro kinase assay. Jurkat cells were suspended in RPMI 1640 medium containing 25 mM HEPES (pH 7.4), incubated for 30 min at 37°C, and stimulated by addition of 5 nM SDF-1α. Cell activation was stopped by adding an equal volume of cold 2× lysis/kinase buffer (100 mM HEPES [pH 7.4], 300 mM NaCl, 1% Nonidet P-40, 10% glycerol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 40 mM NaF, 2 mM Na_3VO_4, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml), and the suspension was incubated on ice for 30 min. After centrifugation, LIMK1 was immunoprecipitated with anti-LIMK1, anti-HA, or anti-Myc antibody and protein A-Sepharose at 4°C for 3 h. The immunoprecipitates were washed three times with 1× lysis/kinase buffer and subjected to an in vitro kinase reaction, as described previously (38).

Immunoblot analysis. Cell lysates or immunoprecipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and incubated with primary antibodies for 1 h at room temperature. After being washed in PBS containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin G (IgG) (Amersham Pharmacia Biotech). Immunoreactive protein bands were visualized by the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech).

Affinity precipitation assays using GST-RBD and GST-PBD. Active GTP-bound forms of Rho family GTPases were detected by affinity precipitation assays, as reported previously (9, 42). Glutathione *S*-transferase (GST) fusion proteins of RBD (GST-RBD) and PBD (GST-PBD) were expressed in *Escherichia coli* BL21 and purified on glutathione-Sepharose, as described previously (35). Jurkat cells were suspended in RPMI 1640 medium containing 25 mM HEPES (pH 7.4), incubated for 30 min at 37°C, and stimulated with 5 nM SDF-1α. For affinity precipitation of Rho, cell activation was stopped by addition of equal volume of 2× lysis/kinase buffer for 20 min on ice. After centrifugation, the supernatant was mixed with 20 μg of GST-RBD bound to glutathione-Sepharose and incubated at 4°C for 1 h. For affinity precipitation of Rac and Cdc42, cell activation was stopped by adding, for 20 min on ice, an equal volume of 2× lysis/kinase buffer containing 8 μg of GST-PBD. After centrifugation, the supernatant was mixed with glutathione-Sepharose and incubated at 4°C for 1 h. The bead pellets were washed three times with 1× lysis/kinase buffer, suspended in 20 μl of Laemmli sample buffer, and analyzed by immunoblotting using an antibody to RhoA, Rac, or Cdc42.

Cell staining. Jurkat cells suspended in RPMI 1640 medium containing 25 mM HEPES (pH 7.4) were incubated for 20 min at 37°C. The cells were then plated on coverslips and allowed to attach for 10 min at 37°C. Attached cells were stimulated with 5 nM SDF-1α for the indicated times and fixed with cold methanol. For blocking we used 5 mg of bovine serum albumin (BSA) per ml in PBS, and then the preparations were incubated with anti-β-actin and anti-P-cofilin antibodies for 1 h at room temperature. After being washed with PBS containing 0.05% Tween 20, the cells were incubated with rhodamine-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG antibodies (Chemicon, Temecula, Calif.) for 30 min at room temperature. For analysis of the effects of S3 peptide, cells suspended in RPMI 1640 medium plus 25 mM HEPES (pH 7.4) were plated on coverslips and incubated for 10 min at 37°C. Attached cells were incubated for 30 min with RPMI 1640 medium containing 25 mM HEPES (pH 7.4) and 0.5% BSA in the presence or absence of 40 μg of S3 or RV peptide per ml, followed by stimulation with 5 nM SDF-1α for 1 min. The cells were fixed, blocked, and stained as described above. Samples were examined using fluorescence microscopy (Leica, Tokyo, Japan).

Chemotaxis assay. Chemotaxis was examined using a 96-well chemotaxis chamber (Neuro Probe, Cabin John, Md.). The lower wells were filled with 32 μl of chemotaxis medium (RPMI 1640 medium containing 0.5% BSA and 25 mM HEPES [pH 7.4]) in the absence or presence of 5 nM SDF-1α with or without 40 μg of S3 or RV peptide per ml and covered with a 5-μm-pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe). Jurkat cells (2×10^5) preincubated for 30 min in chemotaxis medium without SDF-1α in the presence or absence of 40 μg of S3 or RV peptide per ml were loaded onto the upper wells, and the chamber was incubated for 3 h at 37°C. The membrane was fixed in methanol and stained with Diff Quick (Kokusai Shiyaku, Kobe, Japan). The nonmigrating cells on the top of the membrane were removed by wiping and rinsing with water. The membrane was read on a plate reader (Bio-Rad) at 570 nm.

RESULTS

SDF-1α induces activation of LIMK1. Jurkat human leukemic T cells highly express the SDF-1α receptor CXCR4 and display chemotaxis in response to SDF-1α (49). To determine if LIMK1 is involved in SDF-1α-induced chemotactic responses, we stimulated Jurkat cells with SDF-1α and examined the change in the kinase activity of endogenous LIMK1. We used 5 nM SDF-1α in this study, because optimal migration of Jurkat cells was observed at this concentration (data not shown). As shown in Fig. 1A, SDF-1α stimulated the kinase activity of LIMK1 by approximately twofold at 1 min after stimulation, and this induced activation was continued for up to 20 min. SDF-1α also activated LIMK1 in human PBL (Fig. 1B). In contrast to the situation in Jurkat cells, the activation of LIMK1 in PBL was transient, with activity reaching a maximum at 1 min and then reverted to almost basal levels within 20 min after SDF-1α treatment (Fig. 1B). Since activation of chemokine receptors is usually transient and is rapidly terminated by receptor desensitization and internalization (41, 62), our results suggest that in Jurkat cells there is a mechanism which prolongs CXCR4 activation to maintain the sustained activation of LIMK1.

SDF-1α induces cofilin phosphorylation and actin reorganization. Because LIMK1 specifically phosphorylates cofilin to induce actin reorganization (6, 58), we examined the effects of SDF-1α on the actin cytoskeletal organization and the level of cofilin phosphorylation in Jurkat cells. After stimulation with SDF-1α, cells were fixed with methanol and stained with antibodies specific to β-actin and a Ser-3-phosphorylated form of

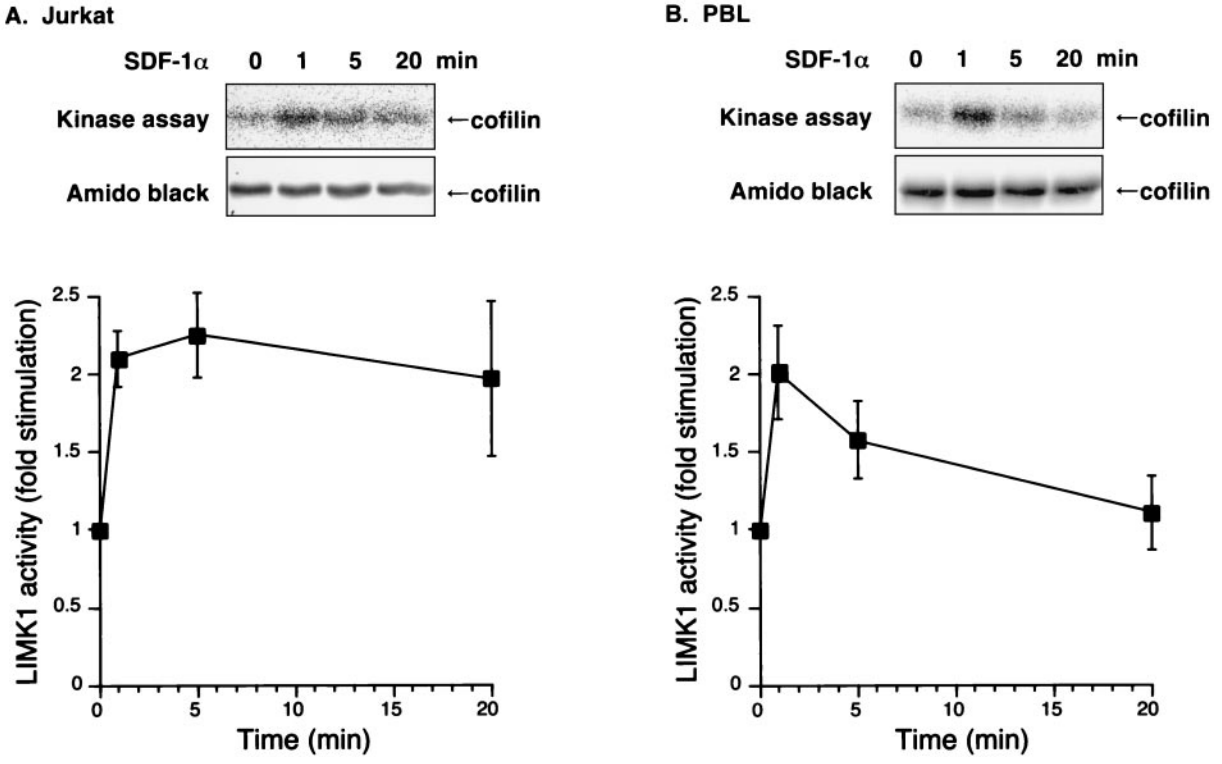

FIG. 1. SDF-1α activates LIMK1 in Jurkat cells and PBL. (A) Jurkat cells were stimulated with 5 nM SDF-1α. At the indicted times, cells were lysed and endogenous LIMK1 was immunoprecipitated and subjected to an in vitro kinase reaction, using His_6-cofilin as a substrate. Reaction mixtures were subjected to SDS-PAGE and analyzed using autoradiography and amido black staining for cofilin. Arrows indicate the position of cofilin. Relative kinase activities of LIMK1 after SDF-1α stimulation are shown as means and standard deviations of three independent experiments, with the value at zero time taken as 1.0. (B) PBL were stimulated with 5 nM SDF-1α, and the kinase activity of endogenous LIMK1 at the indicated times after SDF-1α stimulation was analyzed as in panel A.

cofilin (P-cofilin) (Fig. 2A). The patterns of staining with anti-β-actin antibody in methanol-fixed cells were similar to the findings with phalloidin in paraformaldehyde-fixed cells (data not shown), indicating that most of the actin molecules we observed using anti-β-actin antibody represent filamentous actin (F-actin). We observed a significant increase in the staining of both β-actin and P-cofilin at 1 and 5 min after stimulation (Fig. 2A). Similarly, the increased staining of both β-actin and P-cofilin was observed in Jurkat cells which were transiently transfected with expression plasmid for LIMK1 (data not shown). We next examined the change in the level of phosphorylated cofilin by immunoblotting (Fig. 2B). SDF-1α stimulation for 1 or 5 min resulted in about a fourfold increase in the level of phosphorylated cofilin. These results indicate that SDF-1α rapidly induces actin reorganization and cofilin phosphorylation in Jurkat cells with a time course similar to that seen with LIMK1 activation.

SDF-1α-induced LIMK1 activation is mediated by Gi protein. Chemokine receptors are coupled to heterotrimeric Gi proteins (52). To determine if Gi protein is involved in SDF-1α-induced LIMK1 activation, Jurkat cells were pretreated with the Gi protein inhibitor PTX for 16 h and then stimulated with SDF-1α. The activation of LIMK1 was almost completely abrogated by the PTX pretreatment (Fig. 3A), which suggests that SDF-1α-induced LIMK1 activation is mediated by Gi protein. We next determined if the βγ subunits mediated LIMK1 activation by SDF-1α. Since βARK1ct binds free Gβγ and inhibits the activation of several Gβγ effectors (23), we tested the effects of βARK1ct on the potential for SDF-1α to activate LIMK1 in Jurkat cells. Coexpression of βARK1ct significantly reduced the LIMK1 activation induced by SDF-1α (Fig. 3B), which suggests that βγ subunits of Gi protein are involved in SDF-1α-induced activation of LIMK1 in Jurkat cells.

SDF-1α-induced LIMK1 activation depends on Rac activation. To investigate the possible involvement of Rho family small GTPases in SDF-1α-induced LIMK1 activation in Jurkat cells, we first determined if SDF-1α activates Rho, Rac, and Cdc42. To do this, we used affinity precipitation assays with GST-RBD and GST-PBD, to which only the active GTP-bound forms of Rho and Rac/Cdc42, respectively, specifically bind (9, 42). As shown in Fig. 4A, Rho, Rac, and Cdc42 were all activated by SDF-1α treatment at 1 min after stimulation. The activation lasted for up to 20 min; the time course was similar to that seen with LIMK1 activation. Pretreatment of cells with PTX inhibited the activation of Rac and Cdc42 but not the activation of Rho (Fig. 4B), which suggests that Gi protein mediates the SDF-1α-induced activation of Rac and Cdc42 but not the activation of Rho. Since LIMK1 activation depends on Gi protein, these results suggest that Rac and/or Cdc42 (but not Rho) is involved in the SDF-1α-induced activation of LIMK1.

To further address this issue, we coexpressed LIMK1 with

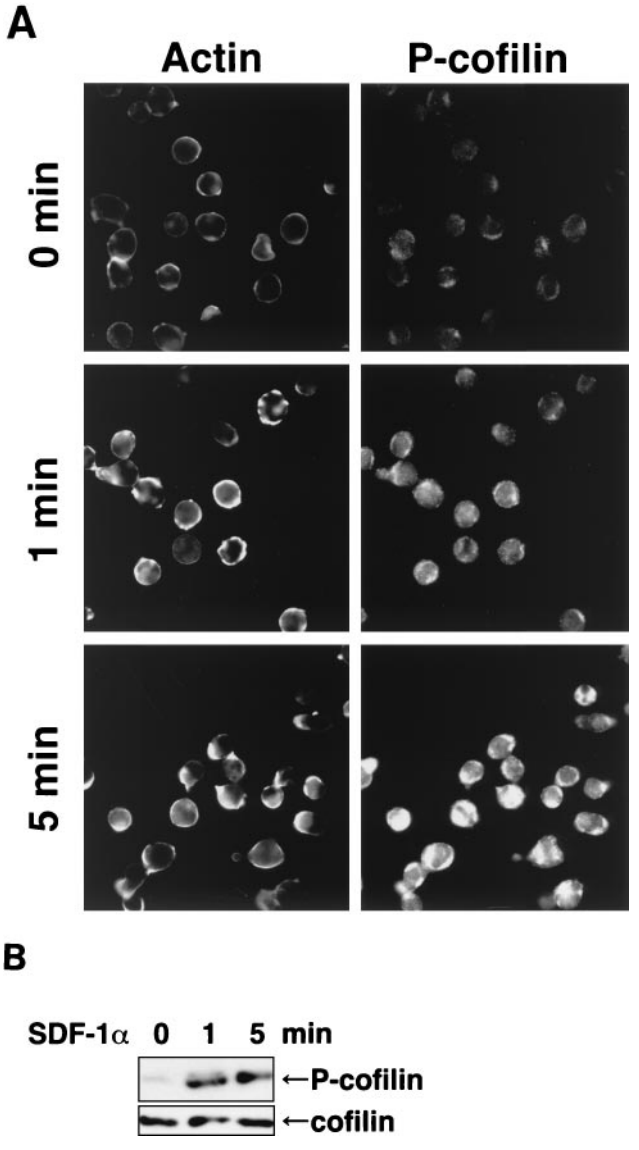

FIG. 2. SDF-1α induces actin reorganization and cofilin phosphorylation in Jurkat cells. (A) Jurkat cells stuck to coverslips were stimulated with 5 nM SDF-1α. At the indicated times, the cells were fixed and stained with anti-β-actin (left) and anti-P-cofilin antibodies (right). (B) Jurkat cells were stimulated with 5 nM SDF-1α. At the indicated times, the cells were lysed and aliquots of total lysates were analyzed by immunoblotting with the antibody against P-cofilin (top) or cofilin (bottom).

any of the dominant negative forms of Rho family small GTPases (RhoN19, RacN17, or Cdc42N17) in Jurkat cells and examined the effect of dominant negative GTPases on SDF-1α-induced LIMK1 activation (Fig. 5). Activation of LIMK1 was abolished by coexpression with RacN17 but not with RhoN19 or Cdc42N17, indicating that Rac, but not Rho or Cdc42, mediates SDF-1α-induced activation of LIMK1. It has been shown that both ROCK and PAK, downstream effectors of Rho and Rac, respectively, activate LIMK1 through direct phosphorylation of LIMK1 at Thr-508 (17, 28, 38). Treatment of cells with Y-27632, a specific inhibitor of ROCK, did not inhibit the LIMK1 activation by SDF-1α (data not shown), which further supports the idea that Rho is not related to the SDF-1α-induced LIMK1 activation. To determine if phosphorylation of LIMK1 at Thr-508 is involved in the SDF-1α-induced activation, we expressed Myc-tagged LIMK1 and its T508V mutant, in which Thr-508 is replaced by a nonphosphorylatable valine residue, in Jurkat cells and determined if SDF-1α would activate Myc-LIMK1 or its T508V mutant (Fig. 6). While the kinase activity of wild-type Myc-LIMK1 was enhanced approximately twofold after SDF-1α treatment, Myc-LIMK1 (T508V) was not activated. These results suggest that the phosphorylation of LIMK1 at Thr-508 is involved in SDF-1α-induced LIMK1 activation and that the Rac-PAK pathway is probably responsible for the phosphorylation.

A cell-permeable peptide inhibitor for LIMK1 blocks SDF-1α-induced cofilin phosphorylation and actin reorganization. To investigate the role of LIMK1-catalyzed cofilin phosphorylation in the SDF-1α-induced actin reorganization and chemotactic response of T cells, we used a cell-permeable synthetic peptide (S3 peptide), which contains the phosphorylation site of cofilin and the cell-permeable sequence motif of penetratin (Fig. 7A) (4). As a control, the RV peptide containing the reverse sequence of cofilin and the penetratin sequence was also tested. We first tested the effects of S3 and RV peptides on the kinase activity of LIMK1 in vitro (Fig. 7B and C). LIMK1 was immunoprecipitated from lysates of SDF-1α-stimulated Jurkat cells and subjected to an in vitro kinase reaction in the absence or presence of S3 or RV peptide, using recombinant cofilin as a substrate. An in vitro kinase assay revealed that the kinase activity of LIMK1 was dose-dependently suppressed by 10 to 40 μg of S3-peptide per ml but not by RV peptide. Thus, S3 peptide has the potential to inhibit the kinase catalytic activity of LIMK1. When Jurkat cells were stimulated with SDF-1α in the presence or absence of S3 or RV peptide and the levels of cofilin phosphorylation and actin remodeling were examined by immunostaining with anti-P-cofilin and anti-β-actin antibodies, cofilin phosphorylation and actin reorganization induced at 1 min after SDF-1α stimulation were evidently inhibited by the addition of S3 peptide (Fig. 8). RV peptide had no apparent effect. These results suggest that S3 peptide inhibits the kinase activity of LIMK1 in Jurkat cells and that LIMK1-catalyzed cofilin phosphorylation is critical for SDF-1α-induced actin reorganization in Jurkat cells.

A cell-permeable peptide inhibitor for LIMK1 blocks SDF-1α-induced chemotaxis. To investigate whether LIMK1-catalyzed cofilin phosphorylation is involved in SDF-1α-induced chemotactic response of T lymphocytes, we examined the effect of S3 peptide on SDF-1α-induced chemotaxis of Jurkat cells. To examine the chemotactic response of Jurkat cells, cells were loaded onto the top wells of a chemotaxis chamber and incubated for 3 h with the bottom wells with or without 5 nM SDF-1α. Cells that migrated into the bottom wells were assessed by staining. As expected, the addition of SDF-1α to the bottom wells increased the chemotactic response of Jurkat cells about threefold (Fig. 9, bars 1 and 2). When Jurkat cells were preincubated with 40 μg of S3 peptide per ml before being loaded, the SDF-1α-induced chemotactic response was inhibited to almost the basal level (bar 3). In contrast, preincubation with RV peptide had no apparent effect (bar 4). These results suggest that cofilin phosphorylation by LIMK1 is essential for SDF-1α-induced chemotaxis in T lymphocytes.

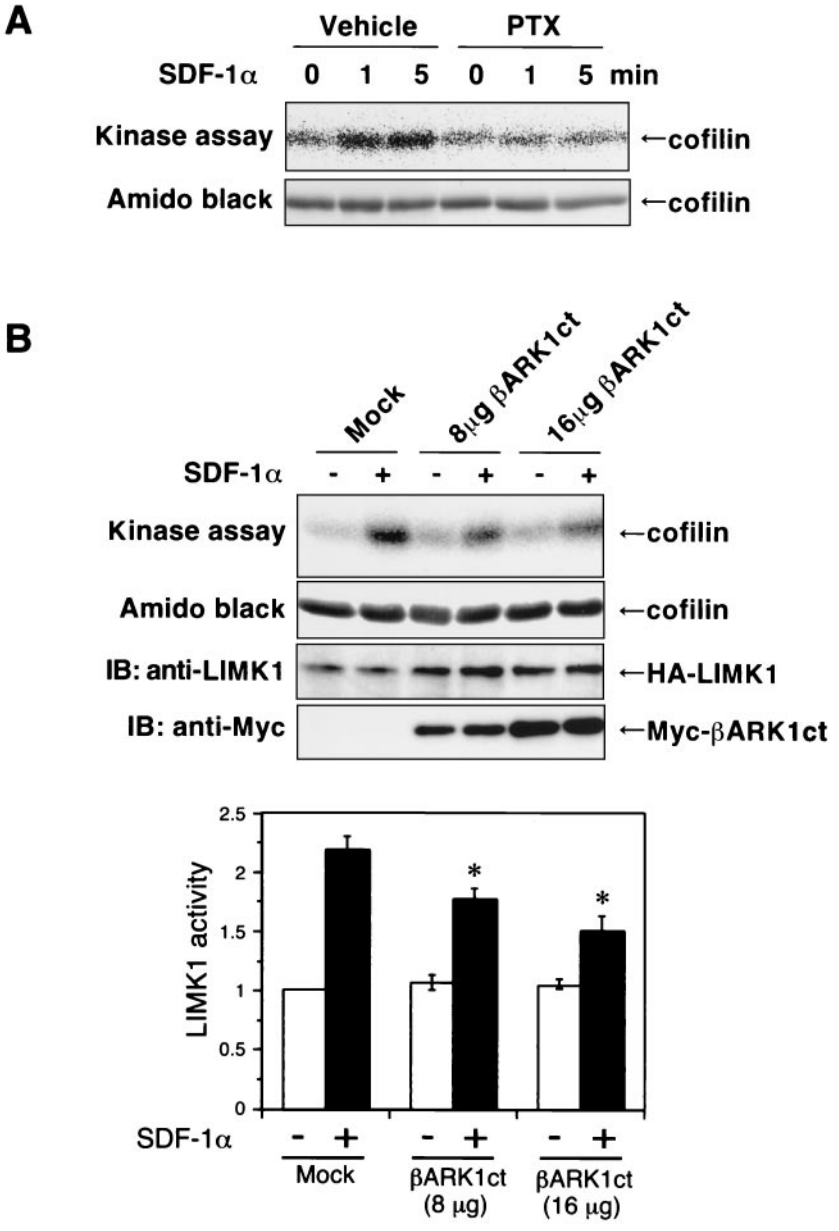

FIG. 3. Effects of PTX and βARK1ct on SDF-1α-induced LIMK1 activation. (A) Jurkat cells were incubated in the presence or absence of PTX (100 ng/ml) for 16 h and then stimulated with 5 nM SDF-1α. At the indicated times after SDF-1α stimulation, the cells were lysed and endogenous LIMK1 was immunoprecipitated and subjected to an in vitro kinase reaction, using His_6-cofilin as a substrate. Reaction mixtures were subjected to SDS-PAGE and analyzed using autoradiography and amido black staining for cofilin. (B) Jurkat cells were cotransfected with expression plasmids coding for HA-LIMK1 and plasmids for Myc-βARK1ct or vector (Mock). The cells were stimulated with 5 nM SDF-1α for 1 min and lysed. HI-LIMK1 was immunoprecipitated with anti-HA antibody and subjected to an in vitro kinase assay, as in panel A. Expression of HA-LIMK1 and Myc-βARK1ct was analyzed by immunoblotting (IB) with anti-LIMK1 and anti-Myc antibodies. The bottom panel indicates the relative LIMK1 kinase activity, with the value of unstimulated Mock cells taken as 1.0. Results are shown as the means and standard deviations of three independent experiments. *, $P < 0.005$ compared with SDF-1α-stimulated mock-transfected cells.

DISCUSSION

Previous studies revealed that LIMK1 has the potential to phosphorylate cofilin and induce actin reorganization and that the kinase activity of LIMK1 is under the control of the Rho-ROCK and Rac-PAK signaling pathways (6, 17, 28, 38, 58). However, the actual role of LIMK1-catalyzed cofilin phosphorylation in cell motility and migration has not been well defined. In the present study we have shown that SDF-1α stimulates the kinase activity of LIMK1 and induces cofilin phosphorylation and actin reorganization in Jurkat T cells. The cell-permeable S3 peptide that inhibits the kinase activity of LIMK1 blocked SDF-1α-induced cofilin phosphorylation, actin reorganization, and the chemotactic response. These results suggest that cofi-

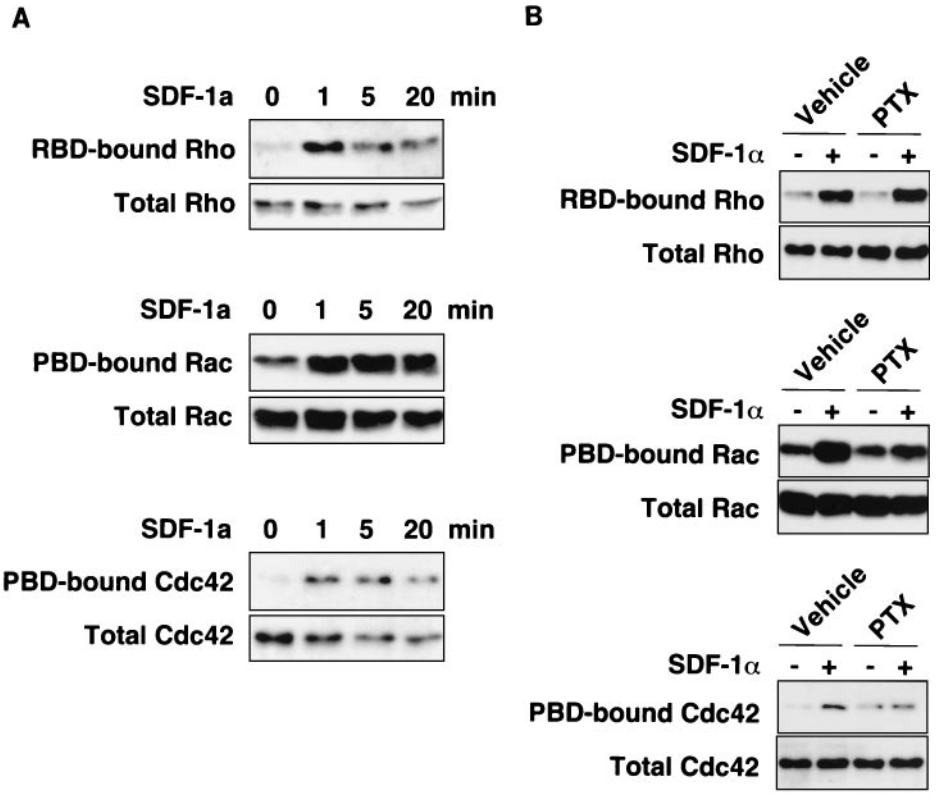

FIG. 4. Activation of Rho, Rac, and Cdc42 by SDF-1α and effects of PTX on their activation. (A) Activation of Rho, Rac, and Cdc42 by SDF-1α. Jurkat cells were stimulated with 5 nM SDF-1α. At the indicated times, the cells were lysed and the lysates were subjected to the affinity precipitation assay for determining Rho, Rac, and Cdc42 activities in the presence of GST-RBD (top) or GST-PBD (middle and bottom). Proteins bound to GST-RBD or GST-PBD and aliquots of total lysates were analyzed by immunoblotting with the antibody against RhoA, Rac, or Cdc42. (B) PTX treatment inhibits SDF-1α-induced activation of Rac and Cdc42 but not Rho. Jurkat cells were pretreated with PTX (100 ng/ml) for 16 h or not pretreated and were then incubated with or without 5 nM SDF-1α for 1 min. Cells were then lysed and subjected to the affinity precipitation assay, as in panel A.

lin phosphorylation by LIMK1 plays a critical role in the SDF-1α-induced actin reorganization and chemotactic response in T lymphocytes.

Cofilin plays an essential role in the rapid turnover of actin filaments by depolymerizing and severing actin filaments (7, 12). The filament-severing activity of cofilin also contributes to the appearance of free barbed ends at the leading edge (12). The activity of cofilin therefore seems to be essential for the lamellipodium and filopodium protrusion at the leading edge of migrating cells (12). Phosphorylation of cofilin at Ser-3 by LIMK1 hinders the interaction between cofilin and actin, thereby inhibiting the actin-depolymerizing and -severing activities of cofilin (15). In fact, overexpression of LIMK1 in cultured cells induces increased levels of cofilin phosphorylation, resulting in inhibition of epidermal growth factor-induced lamellipodium extension and cell motility (60). On the other hand, we have found in the present study that the inhibition of LIMK1-catalyzed cofilin phosphorylation by S3 peptide blocked SDF-1α-induced chemotaxis of T cells. If LIMK1 inactivates cofilin by phosphorylation, why is LIMK1 required for SDF-1α-induced T-cell migration? As previously proposed (46), LIMK1 may play a role in the release of free actin and cofilin from an actin-cofilin dimer complex, which is produced from the pointed end of actin filaments by the action of cofilin. The dissociated P-cofilin can be reused to promote the actin filament turnover after dephosphorylation, although the responsible cofilin phosphatase remains to be determined. If so, LIMK1 may have a function in promoting the turnover of the phosphorylated and dephosphorylated forms of cofilin to support the rapid turnover of actin filaments at the leading edge. In the experiments of LIMK1 overexpression, the cofilin-phosphorylating activity of LIMK1 probably exceeds the endogenous cofilin-phosphatase activity, thereby leading to inactivation of most of cellular cofilin and inhibition of rapid turnover of actin filaments, lamellipodium protrusion, and cell motility. Another possibility is that LIMK1 plays a role in stabilizing actin filaments by inactivating cofilin, since the proper level of stabilization of actin filaments is required for the formation of lamellipodia, fiolopodia, and contractile structures for cell movement.

Previous studies have shown that SDF-1α stimulates several signaling proteins, such as extracellular signal-regulated kinase, protein kinase B, phosphatidylinositol 3-kinase, p21-activated kinase, and Cdc42, in different cell types (18, 49, 53, 57), but the signaling mechanisms by which SDF-1α induces actin reorganization and cell migration remain largely uncharacterized. In the present study, the molecular mechanisms by which SDF-1α activates LIMK1 in Jurkat cells were examined.

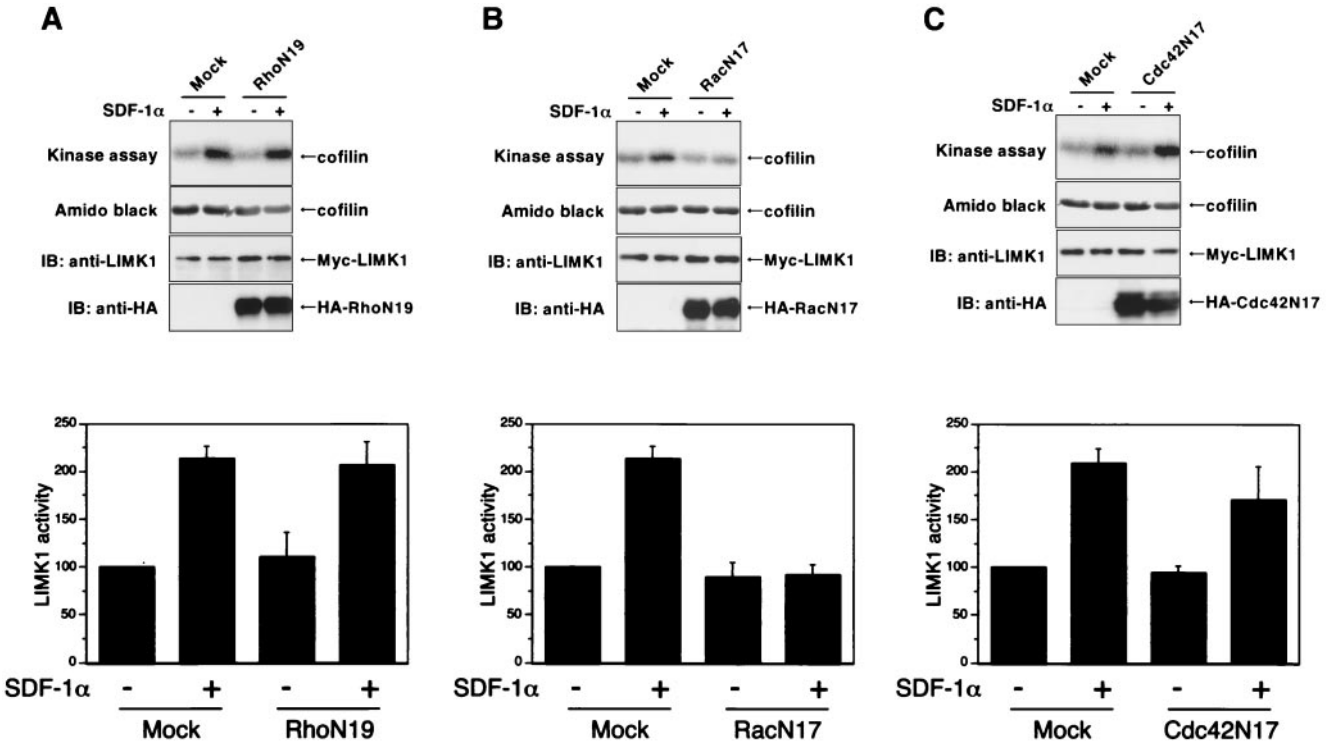

FIG. 5. SDF-1α-induced LIMK1 activation is inhibited by the dominant negative form of Rac but not of either Cdc42 or Rho. Jurkat cells were cotransfected with expression plasmids encoding Myc-LIMK1 and plasmids for RhoN19 (A), RacN17 (B), or Cdc42N17 (C). The cells were stimulated for 1 min with 5 nM SDF-1α and lysed. Myc-LIMK1 was immunoprecipitated with anti-Myc antibody and subjected to an in vitro kinase assay, using His_6-cofilin as a substrate. Expression of Myc-LIMK1 and HA-tagged RhoN19, RacN17, and Cdc42N17 was analyzed by immunoblotting (IB) with anti-LIMK1 and anti-HA antibodies. Results are shown as the means and standard deviations of three independent experiments.

We have shown that SDF-1α-induced LIMK1 activation is sensitive to PTX, indicating that Gi protein is required for this activation. We have also shown that the SDF-1α-induced activation of LIMK1 is suppressed by coexpression of βARK1ct, which inhibits the function of βγ subunits of G proteins (23). These results suggest that LIMK1 activation is mediated by Gi protein βγ subunits in Jurkat cells. On the other hand, since βARK1ct did not completely inhibit LIMK1 activation, it may be that the βγ-independent pathway is also involved in LIMK1 activation, as in the case for ERK that is activated by Gi protein in both βγ-dependent and -independent pathways in Jurkat cells (19).

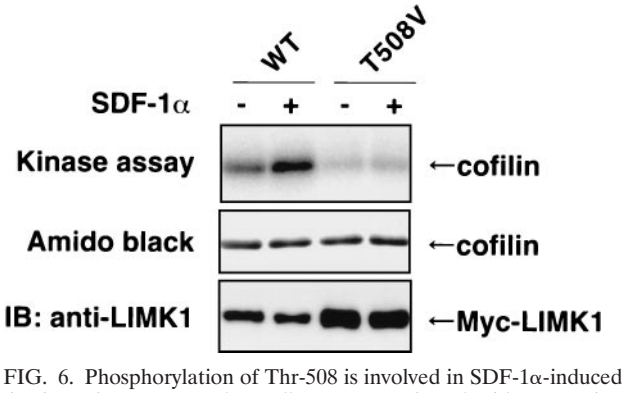

FIG. 6. Phosphorylation of Thr-508 is involved in SDF-1α-induced activation of LIMK1. Jurkat cells were transfected with expression plasmids encoding Myc-LIMK1 or its T508V mutant. The cells were incubated for 1 min with or without 5 nM SDF-1α and lysed. Myc-LIMK1 and its mutant were immunoprecipitated with anti-Myc antibody and subjected to an in vitro kinase assay, using His_6-cofilin as a substrate. Expression of Myc-LIMK1 and its mutant was analyzed by immunoblotting (IB) with anti-LIMK1 antibody. WT, wild type.

We further investigated the possible involvement of Rho family small GTPases in SDF-1α signaling. Rho family small GTPases play a pivotal role in regulating the actin cytoskeleton (8, 24, 37, 43, 44). The best-characterized members of this family are Rho, Rac, and Cdc42. In fibroblasts, Rho regulates the assembly of stress fibers and focal adhesions, whereas Rac and Cdc42 induces the formation of lamellipodia and filopodia, respectively (8, 24, 37, 43, 44). We have shown here that Rho, Rac, and Cdc42 are all activated by SDF-1α, which suggests that these Rho family small GTPases cooperatively regulate the actin cytoskeleton for the SDF-1α-induced chemotactic response of T cells. Activation of Rac and Cdc42 by SDF-1α is sensitive to PTX, but activation of Rho is not, thus indicating that Gi protein mediates the activation of Rac and Cdc42 but not the activation of Rho. In this respect, it is interesting that SDF-1α has the potential to activate the JAK/STAT pathway through CXCR4 in a manner independent of Gi protein (55). Similarly, SDF-1α-induced Rho activation seems to involve a pathway independent of Gi protein.

SDF-1α-induced LIMK1 activation is blocked by the expression of the dominant negative form of Rac but not that of Rho or Cdc42, indicating that LIMK1 activation in Jurkat cells is mediated by Rac but not by either Rho or Cdc42. Since a

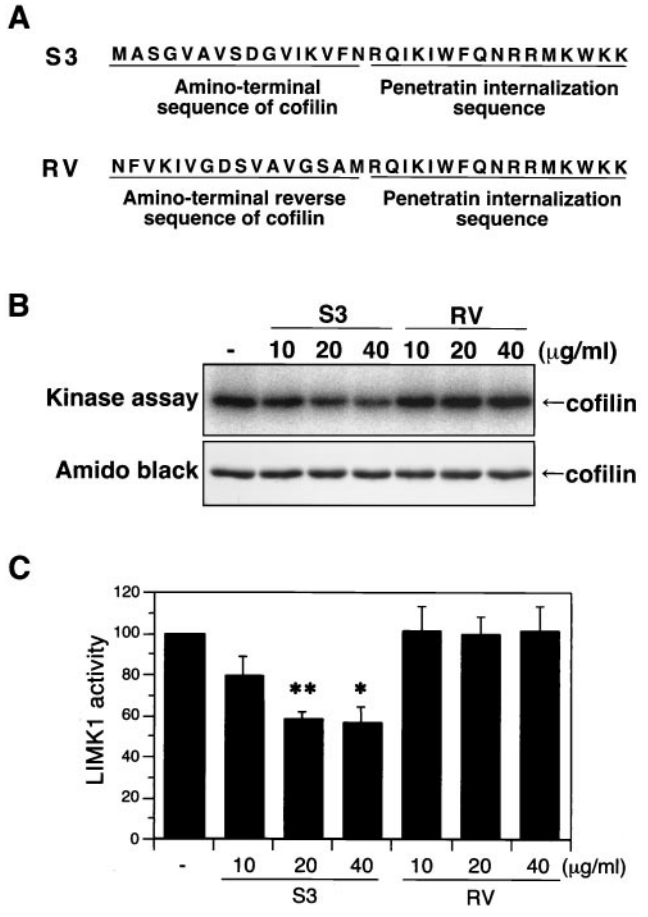

FIG. 7. A cell-permeable S3-peptide, but not RV-peptide, inhibits the kinase activity of LIMK1 in vitro. (A) Structures of S3 and RV peptides. (B) In vitro kinase assay. Jurkat cells were stimulated with 5 nM SDF-1α for 1 min and lysed. LIMK1 was immunoprecipitated with anti-LIMK1 antibody and subjected to an in vitro kinase assay, using His_6-cofilin as a substrate, in the presence or absence of the indicated amounts of S3 or RV peptide. (C) The bands on the autoradiographs were quantified by densitometry. Results are shown as the means and standard deviation of three independent experiments. *, $P < 0.05$ compared with control. **, $P < 0.005$ compared with control.

previous study revealed that Rac activates its effector PAK, which in turn activates LIMK1 through phosphorylation at Thr-508 of LIMK1 (17), LIMK1 is probably activated by Thr-508 phosphorylation by PAK in response to SDF-1α. This is supported by the finding that a LIMK1 mutant in which Thr-508 is replaced by a nonphosphorylatable valine was not activated by SDF-1α. Therefore, it is likely that SDF-1α stimulates the Rac-PAK signaling pathway and thereby induces the phosphorylation and activation of LIMK1.

Recently, Haddad et al. have shown that the interaction of Cdc42 with its effector WASP is needed for SDF-1α-induced chemotaxis of T lymphocytes (18). WASP is the protein encoded by the Wiskott-Aldrich syndrome (WAS) gene and interacts with and activates the actin-related protein 2/3 (Arp2/3) complex to induce actin nucleation and polymerization (20, 21, 26, 27, 45, 59). Thus, SDF-1α stimulates two distinct signaling pathways, the Cdc42-WASP-Arp2/3 and Rac-PAK-LIMK1-cofilin pathways, to induce chemotaxis of T lymphocytes.

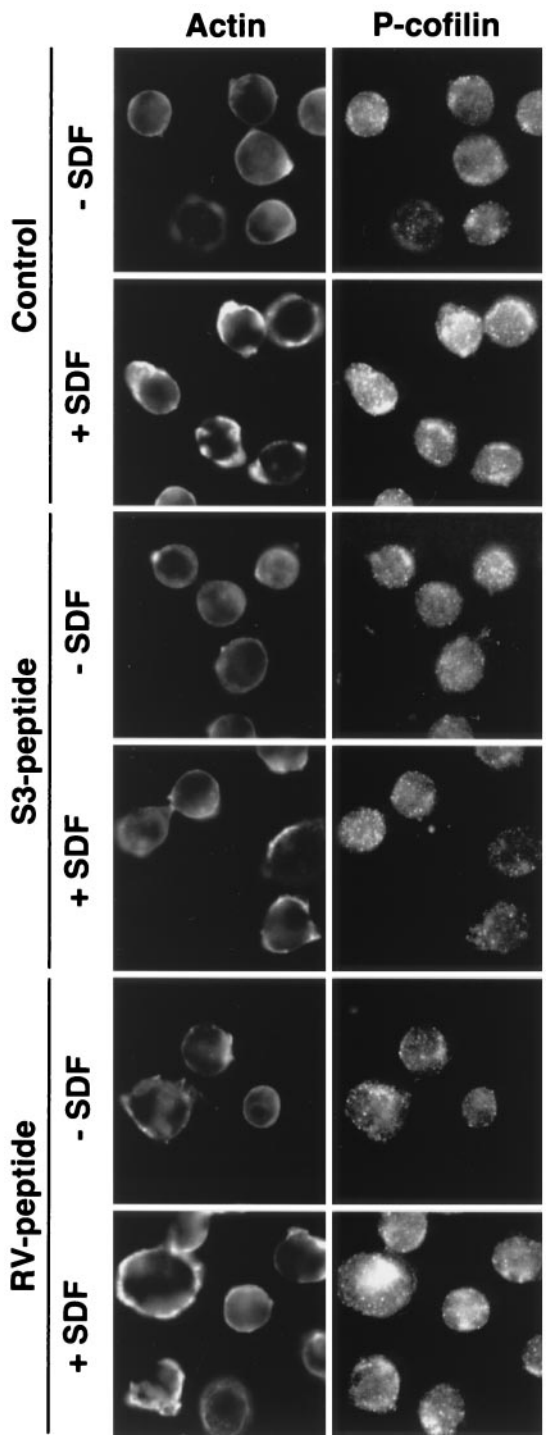

FIG. 8. Effects of S3 and RV peptide on SDF-1α-induced cofilin phosphorylation and actin reorganization in Jurkat cells. Jurkat cells attached to coverslips were preincubated with or without 40 μg of S3 or RV peptide per ml for 30 min and then further incubated with or without 5 nM SDF-1α for 1 min. The cells were fixed and stained with anti-β-actin and anti-P-cofilin antibodies.

Whereas LIMK1-catalyzed cofilin phosphorylation is required for SDF-1α-induced actin reorganization in Jurkat cells (Fig. 8), Cdc42-WASP interaction is dispensable (18). Because Cdc42 plays a critical role in cell polarity formation in various

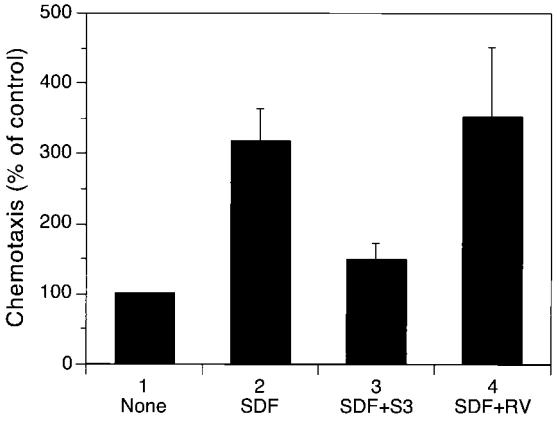

FIG. 9. Effects of S3 and RV peptide on SDF-1α-induced chemotaxis in Jurkat cells. Jurkat cells were preincubated with chemotaxis medium in the presence or absence of 40 μg of S3 or RV peptide per ml for 30 min, and the chemotactic response of Jurkat cells towards 5 nM SDF-1α was determined in a 96-well chemotaxis chamber at 37°C for 3 h, as described in Materials and Methods. Data are expressed as percentages of the control and are shown as the mean and standard deviation of three independent experiments.

cells, including T lymphocytes, macrophages, fibroblasts, and budding yeast (5, 36, 51), the Cdc42-WASP-Arp2/3 pathway probably plays a role in the mechanism of directional movement of cells toward chemotactic signals instead of intrinsic motility of cells. In support of this idea, macrophages isolated from WAS patients show defects in chemotaxis but exhibit random motility indistinguishable from that of wild-type cells (61). Previous studies showed that Rho participates in the chemoattractant-activated signaling pathway to induce integrin-dependent leukocyte adhesion (25). In SDF-1α-induced chemotaxis of T lymphocytes, Rho may play a similar role to regulate integrin-mediated T-cell adhesion to the wall of blood vessels before transmigration across the endothelial cells.

With use of the cell-permeable synthetic S3 peptide that inhibits the in vitro kinase activity of LIMK1, we could block SDF-1α-induced actin reorganization and chemotactic migration of Jurkat cells. Our observations suggest that LIMK1-mediated cofilin phosphorylation plays a critical role in SDF-1α-induced, actin-dependent T-cell migration. Since SDF-1α and its receptor, CXCR4, were also shown to be involved in breast cancer metastasis by directly acting on tumor cell migration (32), LIMK1-catalyzed cofilin phosphorylation is apparently involved in breast cancer metastasis. The potential for the LIMK1-inhibitory peptide as a therapeutic agent which would interfere with tumor cell migration and metastasis warrants further investigations.

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