

Cofilin Phosphorylation and Actin Cytoskeletal Dynamics Regulated by Rho- and Cdc42-activated LIM-kinase 2

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Abstract. The rapid turnover of actin filaments and the tertiary meshwork formation are regulated by a variety of actin-binding proteins. Protein phosphorylation of cofilin, an actin-binding protein that depolymerizes actin filaments, suppresses its function. Thus, cofilin is a terminal effector of signaling cascades that evokes actin cytoskeletal rearrangement. When wild-type LIMK2 and kinase-dead LIMK2 (LIMK2/KD) were respectively expressed in cells, LIMK2, but not LIMK2/KD, phosphorylated cofilin and induced formation of stress fibers and focal complexes. LIMK2 activity toward cofilin phosphorylation was stimulated by coexpression of activated Rho and Cdc42, but not Rac. Importantly, expression of activated Rho and Cdc42, respectively, induced stress fibers and filopodia, whereas both Rho-induced stress fibers and Cdc42-induced filopodia were

abrogated by the coexpression of LIMK2/KD. In contrast, the coexpression of LIMK2/KD with the activated Rac did not affect Rac-induced lamellipodia formation. These results indicate that LIMK2 plays a crucial role both in Rho- and Cdc42-induced actin cytoskeletal reorganization, at least in part by inhibiting the functions of cofilin. Together with recent findings that LIMK1 participates in Rac-induced lamellipodia formation, LIMK1 and LIMK2 function under control of distinct Rho subfamily GTPases and are essential regulators in the Rho subfamilies-induced actin cytoskeletal reorganization.

Key words: cytoskeleton • actin depolymerization • LIM-kinase • Rho family GTPases • stress fibers

CELL movement, division, and shape determination in differentiated cells depend directly on dynamics of the actin cytoskeleton (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). The rapid turnover of actin filaments, i.e., polymerization and depolymerization, and formation of a three-dimensional meshwork during actin cytoskeletal rearrangement are precisely regulated by a variety of actin-binding proteins and proteins involved in severing, capping, and cross-linking of actin filaments (Carlier and Pantaloni, 1997; Ay-scough, 1998). Among them, a group of small (15–22 kD) actin-binding proteins that include cofilin, destrin, depactin, actophorin, and actin depolymerizing factor (ADF)¹,

collectively called ADF/cofilin family, promotes actin depolymerization (Moon and Drubin, 1995). Indeed, cofilin plays a definitive role in actin depolymerization and cofilin is essential for cytokinesis, endocytosis, and other cellular processes that require rapid turnover of actin filaments (Abe et al., 1996; Lappalainen and Drubin, 1997; Theriot, 1997). Cofilin binds to both actin monomers and polymers, and promotes the disassembly of actin filaments. The actin-binding activities of various members of the ADF/cofilin family, including cofilin, are inhibited by protein phosphorylation (Agnew et al., 1995; Moriyama et al., 1996) and/or by competitive binding of phosphoinositides (Yonezawa et al., 1990). Thus, ADF/cofilin members are potential candidates for downstream effectors of several types of signaling cascades that evoke rearrangement of the actin cytoskeleton.

There is mounting evidence that the Rho subfamily of proteins including Rho, Rac, and Cdc42 regulate various forms of the focal adhesion complexes and actin filament dynamics. Rho regulates the formation of actin stress fibers (Ridley and Hall, 1992), whereas Rac regulates assembly of an actin meshwork at the cell periphery, inducing lamellipodia and membrane ruffles (Ridley et al., 1992). Cdc42 regulates filopodial protrusions at the cell

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1. *Abbreviations used in this paper:* ΔLIM, mutant LIM-kinase 2 with a deleted LIM domain; ΔN, mutant LIMK2 with a deleted NH₂-terminal half containing both LIM and PDZ domains; ΔPK, mutant LIMK2 with a deleted kinase domain; ADF, actin depolymerizing factor; cofilin-S3A, constitutively active cofilin; GST, glutathione S-transferase; HA, hemagglutinin; KD, kinase-dead; LIMK, LIM-kinase.

periphery (Kozma et al., 1995; Nobes and Hall, 1995), and all three GTPases also regulate the assembly of focal complexes. Although these actions of the Rho subfamily proteins in the regulation of cell-matrix interaction and the rearrangement of actin cytoskeleton upon extracellular stimuli are well characterized (Takai et al., 1995; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998), biochemical mechanisms by which the Rho subfamily proteins evoke these effects are not as well understood.

Arber et al. (1998) and Yang et al. (1998b) provided evidence that LIM-kinase 1 (LIMK1) specifically phosphorylates cofilin and thus regulates actin filament dynamics, under the control of Rac. This finding provided a clue to links between growth factor-induced activation of Rac and the rapid turnover of actin filaments during lamellipodia formation via protein phosphorylation of the terminal effector protein, cofilin. The data indicate that Rac activates LIMK1, which, in turn, phosphorylates and inactivates cofilin. LIMK is a serine/threonine/tyrosine kinase structurally characterized by NH₂-terminal two LIM domains and PDZ domain. Two closely related LIMKs, i.e., LIMK1 and LIMK2, have been identified (Mizuno et al., 1994; Bernard et al., 1994; Nunoue et al., 1995; Okano et al., 1995; Ikebe et al., 1997; Koshimizu et al., 1997). Sufficient deletion of the gene that codes for LIMK1 is associated with Williams syndrome, a genetic disorder characterized by defects in visuospatial cognition (Frangiskakis et al., 1996). The biological functions of both LIMK1 and LIMK2 heretofore remained unknown.

Here, we provide evidence that LIMK2 phosphorylates cofilin, under control of Rho and Cdc42, but not Rac. Thus, LIMK2 plays a definitive role in regulating stress fiber and filopodia formation and accompanying actin cytoskeletal dynamics, specifically downstream of Rho and Cdc42. Our finding indicates that LIMK2 is a novel downstream effector of Rho and Cdc42 for actin cytoskeletal regulation and that there are at least two distinct pathways in the Rho subfamily-driven actin cytoskeletal rearrangement via cofilin phosphorylation: Rac-LIMK1-cofilin and Rho/Cdc42-LIMK2-cofilin pathways.

Materials and Methods

Materials

Antivinculin mAb (VIN-11-5), anti-FLAG M2 mAb, and TRITC-conjugated anti-rabbit IgG were purchased from Sigma Chemical Co. FITC-conjugated anti-mouse IgG, antihemagglutinin (anti-HA) mAb (12CA5), and anti-Myc mAb (9E10) were purchased from Boehringer Mannheim. Anti-HA (Y-11), anti-Myc (A-14), anti-Cdc42 (SC-87) polyclonal antibody, and rhodamine-conjugated phalloidin were purchased from Santa Cruz Biotechnology and Molecular Probes, Inc. Lipofectamine and Opti-MEM were purchased from GIBCO BRL. Anti-LIMK2 polyclonal antibody was generated as described by Takahashi et al. (1998).

Construction of Expression Plasmids and Preparation of Recombinant Protein

The expression plasmids of Rho subfamily GTPases (pEF-BOS-myc) were constructed as described elsewhere (Komuro et al., 1996). The full-length rat LIMK2 cDNA (Nunoue et al., 1995) was inserted into pBlue-script II SK, using a BamHI linker (pBS-LIMK2). To generate expression plasmids encoding HA-tagged LIMK2 in a pcDNA3 vector (Invitrogen; pcDNA-LIMK2-HA), the cDNA fragment was amplified by PCR using a set of the following primers: forward, 5'-GCGGATCCACCATGGCGC-

GCGCTGGC-3' (I); and the reverse, 5'-GCTCTAGATTAGATATCAGCGTAATCTGGAACATCGTATGGGTAACACTCGAGGGG-TGGGGAGTCCCGGG-3' (the underlined segment corresponds to the antisense sequence coding COOH-terminal 12CA5 HA epitope). cDNA obtained after PCR encodes the full-length rat LIMK2 and subsequent COOH-terminal 12CA5 HA epitope peptide (CYPYDVPDYA). The PCR product digested with BamHI and XbaI was ligated to BamHI- and XbaI-digested pcDNA3 vector. The expression plasmid coding COOH terminally HA-tagged LIMK1 was generated in a similar manner. To generate plasmids encoding ΔLIM-HA (152–638 amino acids), ΔN-HA (329–638 amino acids), and ΔPK-HA (1–326 amino acids), cDNA fragments were amplified by PCR, using the following sets of primers: ΔLIM-HA, the forward primer, 5'-GCGGATCCACCATGCTCATCTCCATGCCGCC-3' (II); and the reverse primer, 5'-GCCTCGAGGGGTGGC-GAGTCCCGGG-3' (III); ΔN-HA, the forward primer, 5'-GCGGATCCACCATGGACCTGATCCACGGGGAG-3'; and the reverse primer, III; ΔPK-HA, the forward primer, I; and the reverse primer, 5'-GCCTCGAGCAGCTGGAAGATCTGCTGTGAGTA-3'. PCR products were digested with BamHI and XhoI and ligated to BamHI- and XhoI-digested pcDNA-LIMK2-HA to replace the original BamHI-XhoI fragment of LIMK2 cDNA with PCR product. The cDNA for kinase defective mutant (LIMK2/KD-HA) was constructed to introduce substitution of the 360th Lys by Met, using a site-directed mutagenesis kit (CLONTECH). To generate the plasmid encoding for glutathione S-transferase (GST)-fused cofilin, full-length mouse cofilin cDNA was cloned by reverse transcriptase PCR, using a set of the following primers: forward primer, 5'-GCGAATTCGAAACATGGCCTCTGGTGTGGC-3' and the reverse primer, 5'-GCGAATTCTCAAAGGCTTGCCTCCAG-3'. The PCR product was digested with EcoRI and ligated to EcoRI-digested pGEX-6P-2 vector (Pharmacia Biotech, Inc.). To generate the expression plasmid encoding for FLAG-tagged cofilin in a pcDNA3 vector (pcDNA-FLAG-cofilin), cDNA fragment was amplified by PCR, using a set of following primers: forward primer, 5'-GCAAGCTTACCATGGACTACAAAGACGATGACGATAAAGGAATTCGAAACATGGCC-3' (the underlined segment correspond to the sense sequence coding FLAG M2 epitope); and the reverse primer 5'-GCTCTAGACTACTCGAGGAATTCTCAAAG-GCTTG-3'. cDNA obtained after PCR encodes the NH₂-terminal FLAG M2 epitope peptide (DYKDDDDK) and subsequent full-length mouse cofilin. The PCR product was digested with HindIII and XbaI and was ligated to HindIII- and XbaI-digested pcDNA3 vector (Invitrogen). The cDNA for constitutively active mutant cofilin (cofilin-S3A) was constructed to introduce substitution of the 3rd Ser by Ala, using a site-directed mutagenesis kit (CLONTECH). The authenticity of the expression plasmids was confirmed by nucleotide sequence analysis. The plasmid coding for GST-fused cofilin was transformed into *Escherichia coli* BL-21. Expression and purification of recombinant protein was carried out using GST Purification Modules (Pharmacia Biotech, Inc.) and the manufacturer's protocol.

Preparation of Antibody

A synthetic peptide, LK1-C15 (RRGESSLPAPHEVDP), corresponding to the COOH-terminal sequence of rat LIMK1, was coupled to keyhole limpet hemocyanin (Sigma Chemical Co.), mixed with Freund's complete adjuvant, and inoculated subcutaneously into rabbits. Anti-LIMK1 antibodies were purified using an antigenic LK1-C15 peptide column, as described (Takahashi et al., 1998).

Transfection and Microinjection

COS-7 cells were maintained in DME supplemented with 10% FBS. HeLa cells were maintained in MEM supplemented with 10% FBS and nonessential amino acids. Subconfluent COS-7 cells were trypsinized, resuspended in PBS, and 10⁶ cells were transfected with 10 μg plasmid DNA by electroporation, using a Gene Pulser (BioRad) according to the manufacturer's instructions. Cells were cultured for 36 h in DME supplemented with 10% FBS.

HeLa cells were plated on a glass coverslip at a density of 6 × 10³/cm² cells and cultured for 12 h and then further cultured for 16 h in serum-free MEM. The cells were transfected in Opti-MEM containing 1 μg plasmid DNA complexed with lipofectamine. After a 2-h incubation, the medium was changed with fresh Opti-MEM and cells were further cultured for 22 h. For microinjection, HeLa cells were plated on a glass coverslip at a density of 6 × 10³/cm² cells and cultured for 12 h. The cells were serum-starved for 24 h in MEM and then plasmid DNA solution (25–100 μg/ml) was microinjected into the nucleus of cells, using an Eppendorf microma-

nipulator system (Eppendorf Scientific, Inc.). Injected cells were cultured for 2–4 h and cells were fixed and stained.

Immunoprecipitation and Protein Kinase Assays

COS-7 cells were transiently transfected with expression plasmid, as described above, and cultured for 36 h. The cells were lysed in 1 ml lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 25 mM β -glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, 1 mM PMSF, 2 $\mu\text{g/ml}$ leupeptin and aprotinin, and incubated on ice for 30 min. After centrifugation, the supernatant was preadsorbed with 15 μl protein G–Sepharose (Pharmacia Biotech, Inc.) for 1 h at 4°C, centrifuged to remove debris, and incubated for 3 h at 4°C with anti-HA antibody (12CA5) and 5 μl protein G–Sepharose. Protein G–Sepharose beads were washed three times with lysis buffer, dissolved in the sample buffer for SDS-PAGE, and subjected to immunoblot analysis, as described (Takahashi et al., 1998).

For protein kinase assay, immunoprecipitates bound to protein G–Sepharose, as described above, were washed three times with kinase buffer consisting of 50 mM Hepes-NaOH, pH 7.5, 25 mM β -glycerophosphate, 5 mM MgCl_2 , 5 mM MnCl_2 , 10 mM NaF, 1 mM Na_3VO_4 , and then incubated for 20 min at 30°C in 15 μl of kinase buffer containing 50 μM ATP, 5 μCi of $\gamma\text{[}^{32}\text{P]ATP}$ (6,000 Ci/mM) and 6 μg of GST-fused cofilin as substrate. After incubating for 20 min at 30°C, the reaction was terminated by heat treatment (100°C for 3 min) in sample buffer for SDS-PAGE and subjected to SDS-PAGE.

Immunofluorescence Analysis

HeLa cells were fixed with 4% paraformaldehyde in PBS for 20 min and treated with PBS containing 0.2% Triton X-100 for 3 min at room temperature. After washing three times with PBS, the cells were incubated with anti-HA, anti-Myc, and anti-FLAG antibodies for 1 h and subsequently with FITC-conjugated anti-mouse IgG and rhodamine-conjugated phalloidin for 1 h. For simultaneous detection of vinculin and HA-tagged LIMKs or Myc-tagged Rho subfamily GTPases, the cells were incubated with mouse antivinculin and rabbit anti-HA or rabbit anti-Myc antibodies for 1 h and then with FITC-conjugated anti-mouse IgG and TRITC-conjugated anti-rabbit IgG for 1 h. The cells were then washed three times with PBS, mounted on glass slides, and then analyzed using a LSM 410 confocal laser scanning microscope (Carl Zeiss).

Results

Distinct Regulation of LIMK1 and LIMK2 by Rho Subfamily GTPases

During a search for substrate proteins of LIMK2, we found that LIMK2 does not phosphorylate myelin basic protein, histone H1, and microtubule associating protein-2, whereas LIMK2 and LIMK1 phosphorylate cofilin, *in vivo* and *in vitro* (data not shown). Based on the finding that cofilin phosphorylation by LIMK1 occurs downstream of Rac (Arber et al., 1998; Yang et al., 1998b), we hypothesized that LIMKs might be regulated by distinct members of Rho subfamily GTPases. To address this issue, HA-tagged LIMK1 or LIMK2 was coexpressed with constitutively active or inactive forms of Rho subfamily GTPases in COS-7 cells, and then LIMK activity was measured using GST-fused cofilin as substrate (Fig. 1). RhoV14, RacV12, and Cdc42V12 are constitutively active forms, while RhoN19, RacN17, and Cdc42N17 are constitutively inactive forms. Consistent with previous reports (Arber et al., 1998; Yang et al., 1998b), LIMK1 activity toward cofilin phosphorylation in cells coexpressing RacV12 or Cdc42V12 was about twofold higher than that seen in control cells expressing LIMK1 alone (Fig. 1 A). Likewise, LIMK1 autophosphorylation was slightly enhanced by coexpression with RacV12 or Cdc42V12 (Fig. 1 A, arrow-

head). However, LIMK1 activity did not significantly change by coexpression of RhoV14 and RhoN19. In fibroblasts and several other cell types, Rho subfamily proteins regulate their activities in a hierarchical cascade wherein Cdc42 activates Rac, which in turn activates Rho (Nobes and Hall, 1995; Allen et al., 1997). Therefore, stimulation of LIMK1 toward cofilin phosphorylation by the active form of Cdc42 might be attributed to the potential of Cdc42 to activate Rac. To examine this possibility, LIMK1 was coexpressed with Cdc42V12 and RacN17, and LIMK1 activity was determined (Fig. 1 B). LIMK1 activity was enhanced to about twofold higher levels by coexpression of Cdc42V12, however, LIMK1 activity enhanced by Cdc42V12 was significantly inhibited by the coexpression of RacN17. This observation indicates that the stimulation of LIMK1 activity by Cdc42 is mediated by the activation of Rac.

In contrast to LIMK1, LIMK2 activity was enhanced to about two- or threefold higher levels by coexpression of either Cdc42V12 or RhoV14 (Fig. 1 C), and autophosphorylation of LIMK2 was also slightly enhanced by coexpression with RhoV14 or Cdc42V12 (Fig. 1 C, arrowhead). However, coexpression of RacV12 did not affect LIMK2 activity, rather, LIMK2 activity was reduced by coexpression of the dominant negative form of Rac (RacN17; Fig. 1 C). Suppression of LIMK2 activity by the coexpression of dominant negative Rac may be due to inhibition of endogenous Rho by dominant negative Rac. These results strongly suggest that protein kinase activities of LIMK1 and LIMK2 are regulated in a distinct manner by distinct members of Rho subfamily GTPases: LIMK1 is regulated by Rac, while LIMK2 is regulated by Rho and Cdc42.

LIMK2 Induces Formation of Actin Stress Fibers and Focal Adhesions

Cofilin promotes depolymerization of actin filaments and phosphorylation of NH_2 -terminal 3rd serine residue results in inactivation of actin-binding and actin-depolymerizing activity of cofilin, events that probably lead to stabilization of the actin cytoskeleton (Agnew et al., 1995; Moriyama et al., 1996). Since LIMK2 phosphorylates cofilin under the control of Rho and Cdc42, we asked if LIMK2 regulates actin cytoskeletal reorganization. HA-tagged LIMK2 was expressed in HeLa cells and the actin cytoskeleton was visualized by making use of rhodamine-conjugated phalloidin (Fig. 2 A). Cells expressing wild-type LIMK2 had an elongated polar fusiform cell shape with pointed edges (Fig. 2 A, e). Importantly, LIMK2 expression markedly enhanced the formation of stress fibers (Fig. 2 A, e and f), and these stress fibers terminated at pointed edges. In these cells, LIMK2 was mainly localized to the cytoplasm and peripheral membranous areas, and was strongly enriched at pointed edge areas, which overlapped with the accumulated actin filaments. The colocalization of LIMK2 and actin filaments strongly suggests that LIMK2 participates in regulating or maintaining the actin filaments. Moreover, when focal adhesion complexes were visualized by vinculin staining, focal adhesion complexes in cells expressing wild-type LIMK2 clearly increased both in size and number, and thus became denser (Fig. 2 B, e and f). However, these characteristic changes

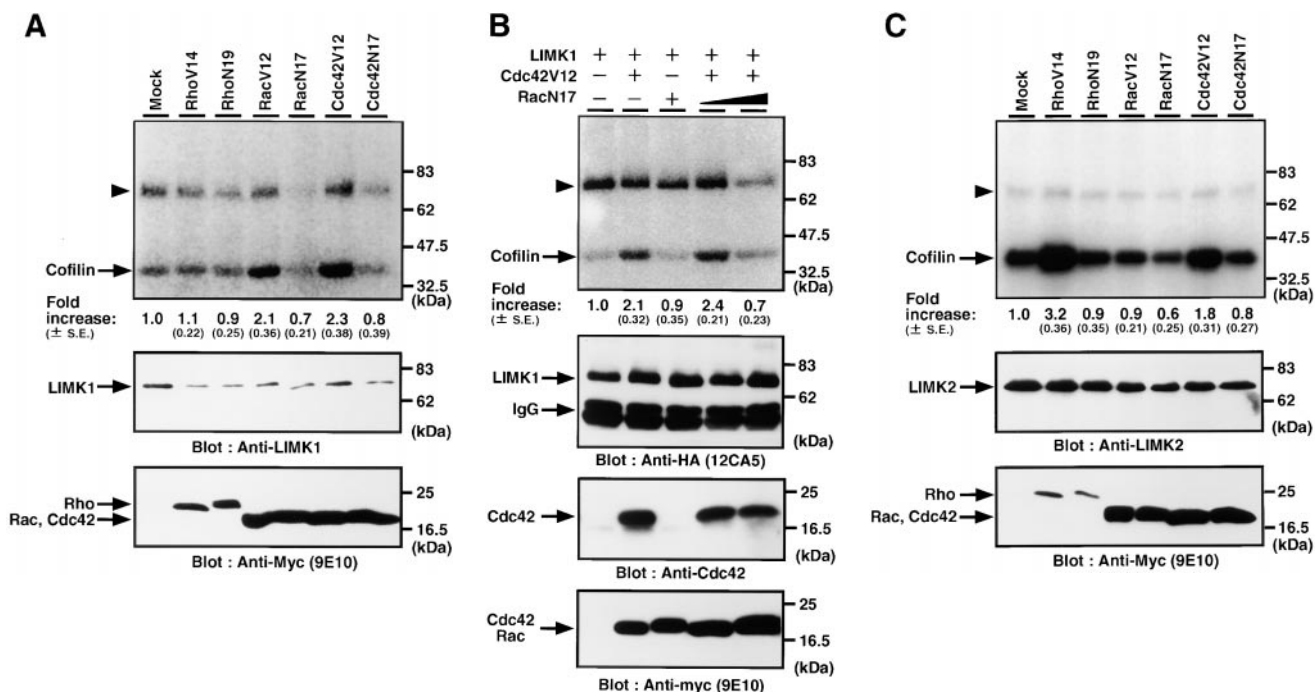


Figure 1. Activation of LIMK1 (A and B) and LIMK2 (C) kinase activity by Rho family GTPases. A, COS-7 cells were coexpressed with HA-tagged full-length LIMK1, and empty vector (Mock), Myc-tagged active, or the inactive form of Rho, Rac, and Cdc42, respectively. B, COS-7 cells were coexpressed with HA-tagged LIMK1 and empty vector (Mock), Myc-tagged Cdc42V12, RacN17, respectively, or increasing doses of Myc-tagged RacN17 (5, 10 μ g), together with HA-tagged LIMK1 and Myc-tagged Cdc42V12. C, COS-7 cells were coexpressed with HA-tagged LIMK2 and empty vector (Mock), Myc-tagged active, or the inactive form of Rho, Rac, and Cdc42, respectively. After transient expression, LIMK1 and LIMK2 were respectively immunoprecipitated from cell lysates, using anti-HA antibody, run on SDS-PAGE, and immunoblotted with anti-LIMK1, anti-LIMK2, and anti-HA antibody. The immunoprecipitates were subjected to *in vitro* kinase reaction with γ - 32 PATP, using GST-fused cofilin as substrate. The phosphorylated cofilin was visualized by autoradiography after SDS-PAGE. The initial extracts (50 μ g) were also subjected to anti-Myc or anti-Cdc42 immunoblot. The arrowheads respectively indicate autophosphorylated LIMK1 or LIMK2. Cofilin phosphorylation was estimated using an image analyzer (model BAS-2000; Fuji), and the amount of cofilin phosphorylation by LIMK1 or LIMK2 expressed with Mock was respectively taken as 1.0. Each value represents the mean \pm standard error (SE) of three independent experiments.

in cell shape, stress fibers, and focal adhesion complexes were absent in cells expressing protein kinase-dead LIMK2 (LIMK2/KD; Fig. 2, A and B, g and h), which did not phosphorylate cofilin (see Fig. 7 B). Similar results were obtained when HA-tagged LIMK1 was expressed in HeLa cells. The formation of actin stress fibers and focal adhesion complexes was induced by expression of LIMK1 (Fig. 2, A and B, a and b), whereas LIMK1/KD failed to induce stress fibers and focal adhesion complexes (Fig. 2, A and B, c and d). These results indicate that LIMK2 (as well as LIMK1) is likely to play an important role in actin cytoskeletal reorganization and in assembly of focal adhesion complexes, and that effects of LIMKs on the cytoskeletal reorganization depend on its protein kinase activity.

We attempted to determine if the effects of LIMK2 on the actin cytoskeleton and focal adhesion complexes are mediated by the inactivation of cofilin. As shown in Fig. 3, expression of cofilin-S3A (substitution of 3rd Ser by Ala) had no apparent effect on actin filaments (Fig. 3 A, c and d) and on focal adhesion complexes in serum-starved HeLa cells (data not shown). Expression of LIMK2 markedly induced formation of stress fibers (Fig. 3 A, a and b), whereas coexpression of cofilin-S3A with LIMK2 significantly repressed LIMK2-induced stress fiber formation

(Fig. 3 A, e and f); stress fibers were induced in 90% of cells expressing LIMK2 alone (130 out of 145 LIMK2 positive cells), but only in 10% of coinjected cells (15 out of 150 LIMK2 positive cells). Furthermore, coexpression of cofilin-S3A with LIMK2 significantly inhibited the LIMK2-induced formation of focal adhesion complexes (Fig. 3 B); focal adhesion complexes were induced in 95% of cells expressing LIMK2 alone (Fig. 3 B, a and b; 135 out of 142 LIMK2 positive cells), but only in 30% of coexpressed cells (Fig. 3 B, c and d; 45 out of 150 LIMK2 positive cells). Similar results were obtained when the cells were coexpressed with LIMK1 and cofilin-S3A (data not shown). These results suggest that LIMK2, as well as LIMK1, inhibits the activity of cofilin through phosphorylation of the serine residue at position 3 and probably leads to stabilization of the actin filaments and to assembly of focal adhesion complexes.

Definitive Role of LIMK2 in Rho- or Cdc42-mediated Actin Cytoskeletal Reorganization

Based on results of LIMK1- and LIMK2-induced actin cytoskeletal reorganization and focal adhesion complexes, as well as distinct regulation of protein kinase activity by Rho

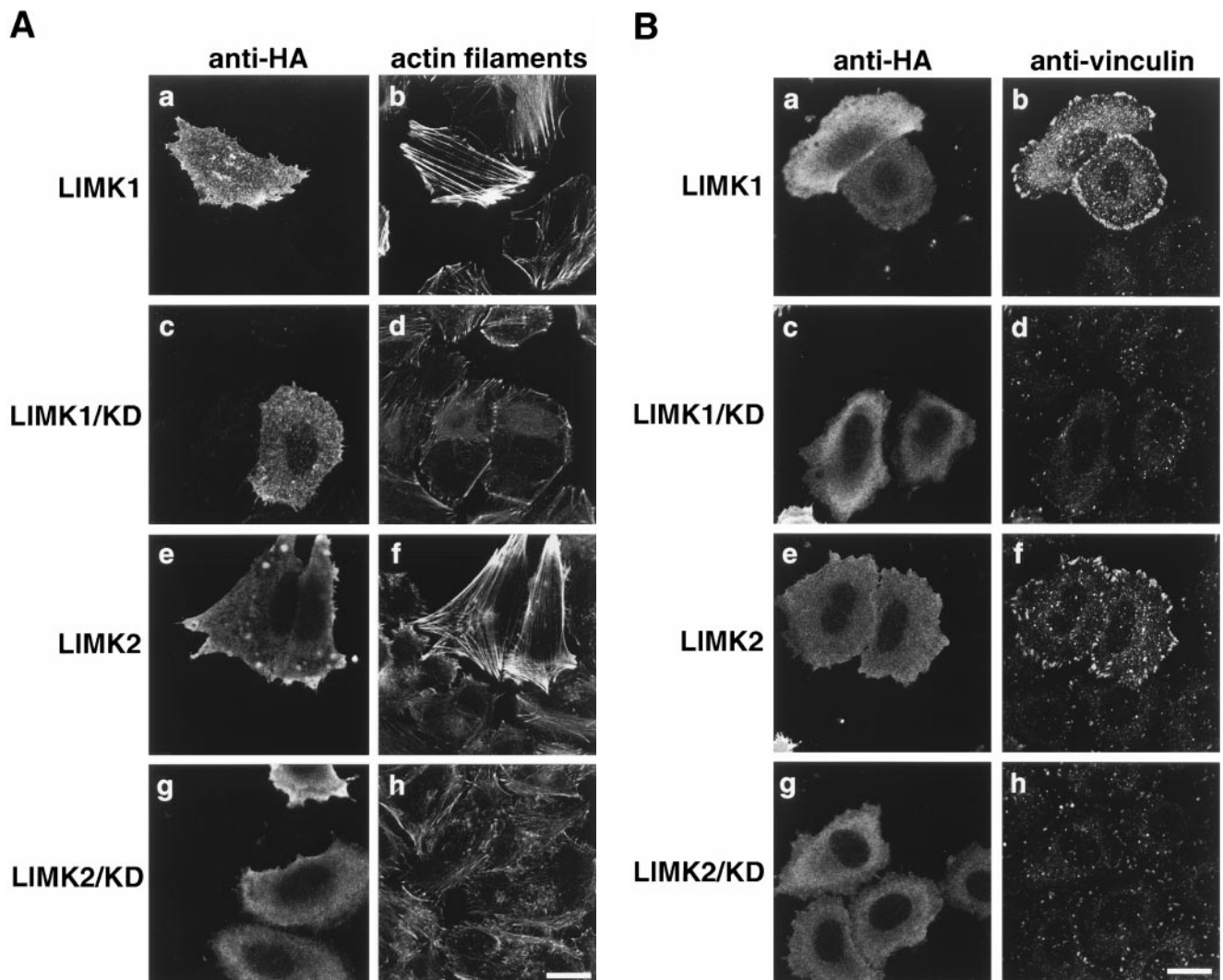


Figure 2. Formation of stress fibers (A) and focal complexes (B) by LIMK1 and LIMK2 in HeLa cells. Serum-starved HeLa cells were transfected with DNA construct encoding HA-tagged full-length LIMK1 (a and b), LIMK2 (e and f), LIMK1/KD (c and d), or LIMK2/KD (g and h). A, The cells were fixed and double stained with anti-HA antibody (a, c, e, and g) and phalloidin (b, d, f, and h). B, The cells were fixed and double stained with anti-HA antibody (a, c, e, and g) and antivinculin antibody (b, d, f, and h). Confocal images were obtained, as described in Materials and Methods. The results shown are representative of three independent experiments. Bars, 20 μ m.

subfamily GTPases, we then determined if LIMK1 and LIMK2 exhibit specific functions on the actin cytoskeletal changes induced by Rho, Rac, and Cdc42 (Fig. 4). Expression of active forms of Cdc42 (Cdc42V12), Rho (RhoV14), and Rac (RacV12) in HeLa cells, respectively, induced distinct cytoskeletal changes such as filopodia (Fig. 4 A, a and b), stress fibers (Fig. 4 B, a and b), and lamellipodia (Fig. 4 C, a and b). Consistent with the notion that Cdc42V12 transiently induces filopodia (Kozma et al., 1995; Nobes and Hall, 1995), the filopodia induced by Cdc42V12 were replaced by lamellipodia within 4 h after the microinjection of plasmid, and filopodia formation occurred in 30% of cells expressing Cdc42V12 (46 out of 153 Cdc42V12 positive cells). The replacement of filopodia by lamellipodia in Cdc42V12 positive cells is due to the potential of Cdc42 to activate Rac (Kozma et al., 1995; Nobes and Hall, 1995). Importantly, coexpression of LIMK2/KD

with Cdc42V12 significantly inhibited Cdc42V12-induced filopodia formation (Fig. 4 A, e and f); filopodia formation was induced only in 6% of coinjected cells (9 out of 145 LIMK2/KD positive cells). Furthermore, LIMK2/KD strongly inhibited RhoV14-induced stress fiber formation (Fig. 4 B, e and f). Stress fiber formation was induced in 96% of cells expressing RhoV14 alone (149 out of 155 RhoV14 positive cells), whereas it was induced only in 15% of coinjected cells (21 out of 142 LIMK2/KD positive cells). The remaining cells have an actin cytoskeleton characterized by a marked reduction in stress fibers and a loss of straight and directed alignment of these fibers. Interestingly, although LIMK2/KD inhibited RhoV14-induced stress fiber formation, it did allow for actin accumulation and partial actin polymerization in regions underlying plasma membranes, as induced by RhoV14. Rho-associated kinase (Rho-kinase) was reported to phosphorylate

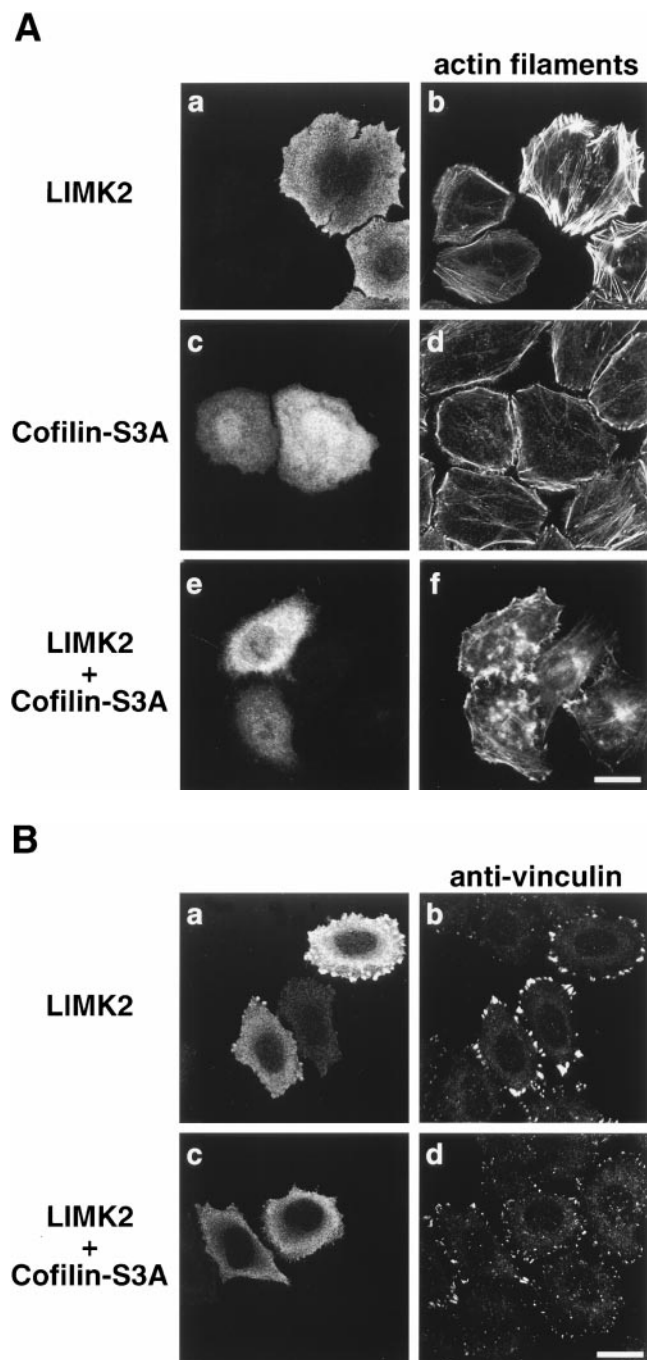


Figure 3. Inhibition of LIMK2-induced actin reorganization (A) and focal complex formation (B) by cofilin inactivation. Expression vectors for FLAG-tagged cofilin-S3A (100 μ g/ml) and HA-tagged LIMK2 (25 μ g/ml) were microinjected into serum-starved HeLa cells. A, 2 h later, cells were fixed and double stained with anti-FLAG antibody (c), anti-HA antibody (a and e), and phalloidin (b, d, and f). B, Two hours later, cells were fixed and double stained with anti-HA antibody (a and c) and anti-vinculin antibody (b and d). Confocal images were obtained as described in Materials and Methods. The results shown are representative of three independent experiments. Bars, 20 μ m.

ERM proteins (ezrin, radixin, and moesin), thereby resulting in cross-linking of actin filaments to plasma membranes (Matsui et al., 1998). Therefore, Rho-induced actin polymerization and/or recruitment of actin filaments in

close proximity to plasma membranes may possibly be regulated by a distinct mechanism other than the LIMK2-cofilin pathway, and LIMK2 may also be involved in Rho-induced bundling and/or cross-linking of actin filaments.

On the other hand, expression of RacV12 induced both lamellipodia and stress fibers that were less prominent than Rho-induced stress fibers (Fig. 4 C, a and b). The co-expression of LIMK2/KD with RacV12 had no apparent effect on RacV12-induced lamellipodia formation, while Rac-induced stress fiber formation was markedly reduced by the coexpression of LIMK2/KD (Fig. 4 C, e and f). Since Rac induces formation of lamellipodia, concomitant with Rho-dependent stress fiber formation (Ridley et al., 1992), the inhibition of Rac-induced weak stress fiber formation by LIMK2/KD may be due to the inhibition of endogenous Rho-induced stress fiber formation. In contrast, consistent with previous reports (Arber et al., 1998; Yang et al., 1998b), coexpression of LIMK1/KD with RacV12 significantly inhibited Rac-induced lamellipodia (Fig. 4 C, c and d), whereas the Rac-induced cell spreading and stress fiber formation were not affected. Lamellipodia formation was induced in 85% of cells expressing RacV12 alone (140 out of 165 RacV12 positive cells), whereas it was induced only in 25% of coinjected cells (38 out of 153 LIMK1/KD positive cells). These results again strongly suggest that LIMK2 is functioning downstream of Rho, but not Rac, whereas LIMK1 is functioning downstream of Rac, but not Rho. On the other hand, in the case of Cdc42- and Rho-induced actin reorganization, coexpression of LIMK1/KD had no apparent effect on Cdc42V12-induced filopodia (Fig. 4 A, c and d) and Rho-induced stress fiber formation (Fig. 4 B, c and d). It was reported that in the presence of dominant negative Rac, Cdc42 induced sustained formation of filopodia (Nobes and Hall, 1995), however, sustained filopodia were not induced in Cdc42V12 and LIMK1/KD coexpressed cells. Together with the finding that LIMK1/KD allowed for Rac-induced cell spreading, LIMK1 is involved in Rac-induced lamellipodia, but may not play a role in other Rac-induced actin cytoskeletal reorganization.

We next examined the effect of LIMK1/KD and LIMK2/KD on the formation of focal adhesion complexes induced by Rho subfamily GTPases. Expression of the active form of Cdc42 (Cdc42V12) in HeLa cells induced formation of focal adhesion complexes (Fig. 5 A, a and b); focal adhesion complexes were induced in 70% of cells expressing Cdc42V12 alone (106 out of 152 Cdc42V12 positive cells). The coexpression of LIMK2/KD with Cdc42V12 significantly decreased size of the focal adhesion complexes (Fig. 5 A, e and f); focal adhesion complexes were induced in 27% of coinjected cells (40 out of 148 LIMK2/KD positive cells). Similar results were observed when LIMK2/KD was coexpressed with the active form of Rho (RhoV14). Focal adhesion complexes were induced in 95% of cells expressing RhoV14 alone (Fig. 5 B, a and b; 157 out of 165 RhoV14 positive cells), while coexpression of LIMK2/KD significantly decreased size and number of focal adhesion complexes (Fig. 5 B, e and f); focal adhesion complexes were induced in 46% of the coinjected cells (67 out of 145 LIMK2/KD positive cells). In contrast, coexpression of LIMK2/KD and RacV12 had no

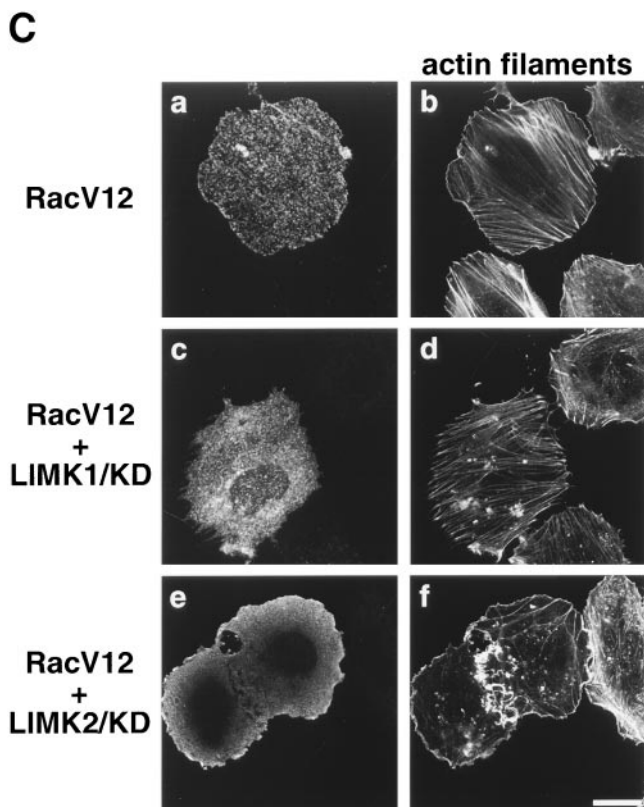
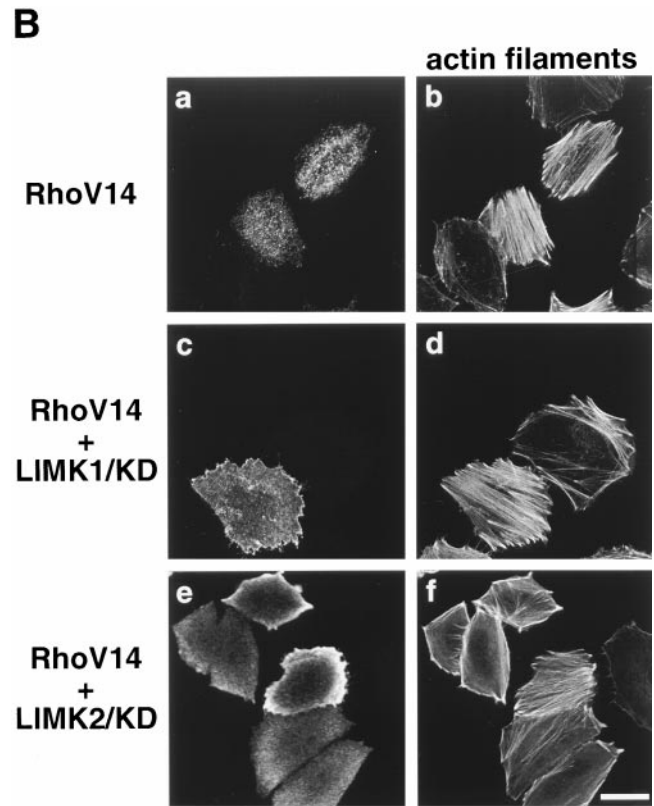
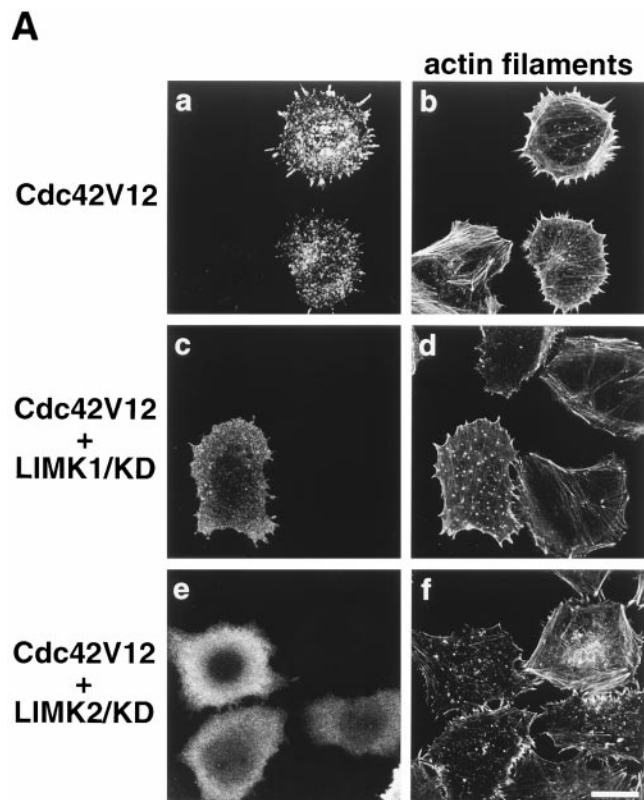


Figure 4. Specific role of LIMK1 and LIMK2 in actin reorganization induced by Rho family GTPases. **A**, Expression vectors for HA-tagged LIMK1/KD or LIMK2/KD (50 μ g/ml) and Myc-tagged active form of Cdc42 (Cdc42V12; 50 μ g/ml) were microinjected into serum-starved HeLa cells. **B**, Expression vectors for HA-tagged LIMK1/KD or LIMK2/KD (50 μ g/ml) and the Myc-tagged active form of Rho (RhoV14; 50 μ g/ml) were microinjected into serum-starved HeLa cells. **C**, Expression vectors for HA-tagged LIMK1/KD or LIMK2/KD (50 μ g/ml) and Myc-tagged active form of Rac (RacV12; 50 μ g/ml) were microinjected into serum-starved HeLa cells. 4 h later, these cells were fixed and double stained with anti-HA antibody (c and e), anti-Myc antibody (a), and phalloidin (b, d, and f). Confocal images were obtained as described in Materials and Methods. The results shown are representative of three independent experiments. Bars, 20 μ m.

apparent effect on formation of RacV12-induced focal adhesion complexes (Fig. 5 C, e and f).

In contrast to LIMK2/KD, coexpression of LIMK1/KD and RacV12 significantly reduced the size of Rac-induced

focal adhesion complexes (Fig. 5 C, c and d); focal adhesion complexes were induced in 90% of cells expressing RacV12 alone (Fig. 5 C, a and b; 139 out of 155 RacV12 positive cells), whereas complexes were induced in 30%

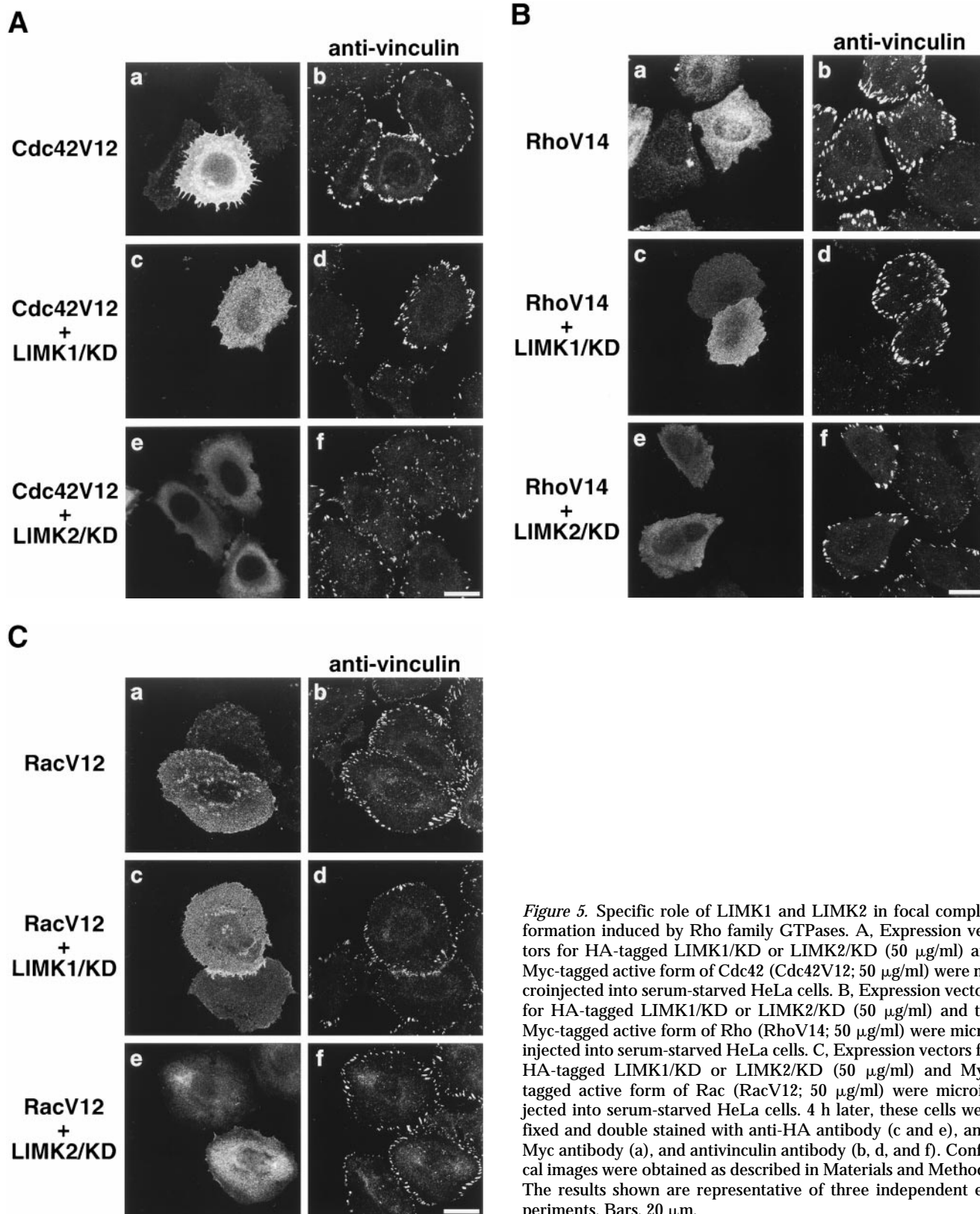


Figure 5. Specific role of LIMK1 and LIMK2 in focal complex formation induced by Rho family GTPases. **A**, Expression vectors for HA-tagged LIMK1/KD or LIMK2/KD (50 μ g/ml) and Myc-tagged active form of Cdc42 (Cdc42V12; 50 μ g/ml) were microinjected into serum-starved HeLa cells. **B**, Expression vectors for HA-tagged LIMK1/KD or LIMK2/KD (50 μ g/ml) and the Myc-tagged active form of Rho (RhoV14; 50 μ g/ml) were microinjected into serum-starved HeLa cells. **C**, Expression vectors for HA-tagged LIMK1/KD or LIMK2/KD (50 μ g/ml) and Myc-tagged active form of Rac (RacV12; 50 μ g/ml) were microinjected into serum-starved HeLa cells. 4 h later, these cells were fixed and double stained with anti-HA antibody (c and e), anti-Myc antibody (a), and anti-vinculin antibody (b, d, and f). Confocal images were obtained as described in Materials and Methods. The results shown are representative of three independent experiments. Bars, 20 μ m.

of the coinjected cells (42 out of 142 LIMK1/KD positive cells). However, coexpression of LIMK1/KD and Cdc42V12 or RhoV14 had no apparent effect on Cdc42V12- and Rho-induced focal adhesion complex formation

(Fig. 5, A and B, c and d). Thus, LIMK1/KD and LIMK2/KD specifically inhibits Rho subfamily GTPases-induced actin cytoskeletal reorganization and focal adhesion complexes, presumably acting as a dominant negative form.

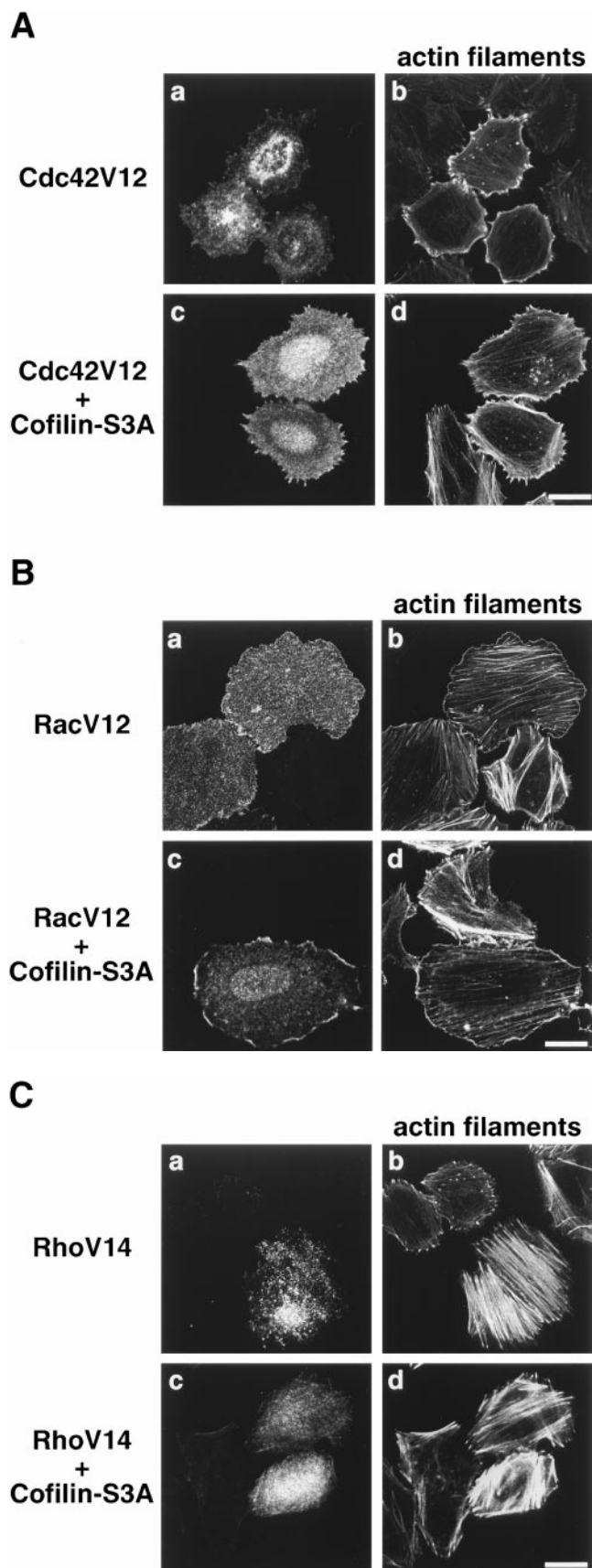


Figure 6. Effect of the active form of cofilin on actin reorganization induced by Rho family GTPases. **A**, Expression vectors for FLAG-tagged cofilin-S3A (50 μ g/ml) and the Myc-tagged active

All these observations strongly suggest that LIMK1 and LIMK2 regulate actin cytoskeletal reorganization and assembly of focal adhesion complexes, under control of Rho subfamily GTPases.

Effect of Overexpression of Cofilin-S3A Mutant on Rho Subfamily GTPases-induced Actin Reorganization

Since LIMKs/KD inhibited Rho subfamily GTPases-induced cytoskeletal reorganization and cytoskeletal reorganization induced by the overexpression of LIMKs was inhibited by the constitutively active form of cofilin, we next determined if the cytoskeletal changes induced by activated Cdc42, Rho, and Rac were influenced by expression of the active form of cofilin. Cofilin-S3A was coexpressed with the active forms of Cdc42, Rac, and Rho in HeLa cells. Expression of the active forms of Cdc42, Rac, and Rho, respectively, induced distinct cytoskeletal changes characterized by filopodia (Fig. 6 A, a and b), lamellipodia (Fig. 6 B, a and b), and stress fibers (Fig. 6 C, a and b). However, coexpression of cofilin-S3A with Rho subfamily GTPases had no apparent effect on Cdc42V12-induced filopodia (Fig. 6 A, c and d), RacV12-induced lamellipodia (Fig. 6 B, c and d), and RhoV14-induced stress fiber formation (Fig. 6 C, c and d) in our system. Likewise, coexpression of cofilin-S3A and Rho subfamily GTPases had no apparent effect on focal adhesion complexes induced by these Rho subfamily GTPases (data not shown).

Functional Domain in LIMK2

To elucidate the function of each domain in LIMK2 in Rho- and Cdc42-mediated cellular processes, deleted mutants of LIMK2 were expressed in COS-7 cells (Fig. 7 A) and we examined their protein kinase activity, subcellular localization, and ability to induce actin cytoskeletal reorganization. As shown in Fig. 7 B, wild-type (full-length) LIMK2 exhibited autophosphorylation and kinase activity toward cofilin, whereas LIMK2/KD did not phosphorylate cofilin. Deletion of the NH₂-terminal half containing both LIM and PDZ domains (Δ N) resulted in reduced protein kinase activity, indicating that the NH₂-terminal half appears to be important for kinase activity. However, the kinase activity of a mutant with a deleted LIM domain (Δ LIM) was about threefold higher than that of wild-type LIMK2, indicating that the LIM domains of LIMK2 appear to behave as negative regulatory domains.

We next analyzed the functional domains in LIMK2 responsible for the induction of actin cytoskeletal reorgani-

form of Cdc42 (Cdc42V12; 50 μ g/ml) were microinjected into serum-starved HeLa cells. **B**, Expression vectors for FLAG-tagged cofilin-S3A (50 μ g/ml) and the Myc-tagged active form of Rac (RacV12; 50 μ g/ml) were microinjected into serum-starved HeLa cells. **C**, Expression vectors for FLAG-tagged cofilin-S3A (50 μ g/ml) and the Myc-tagged active form of Rho (RhoV14; 50 μ g/ml) were microinjected into serum-starved HeLa cells. 4 h later, these cells were fixed and double stained with anti-Myc antibody (a), anti-FLAG antibody (c), and phalloidin (b and d). Confocal images were obtained as described in Materials and Methods. The results shown are representative of three independent experiments. Bars, 20 μ m.

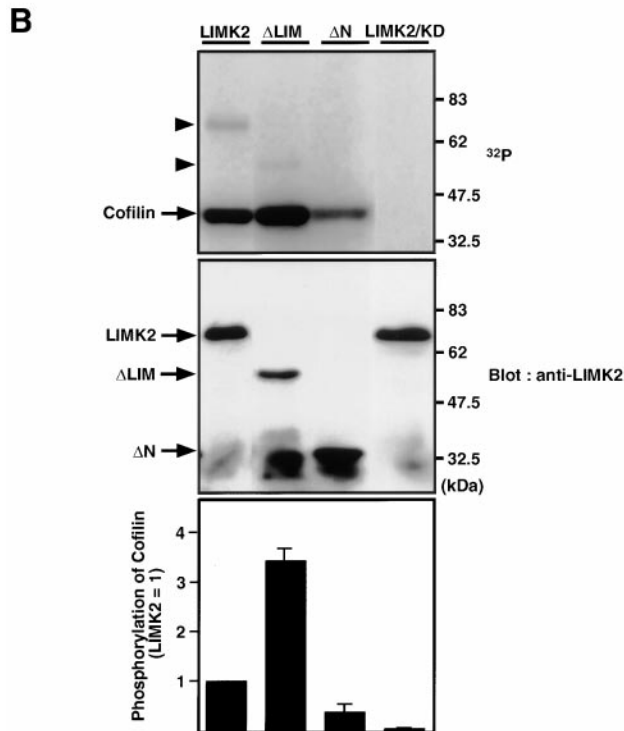
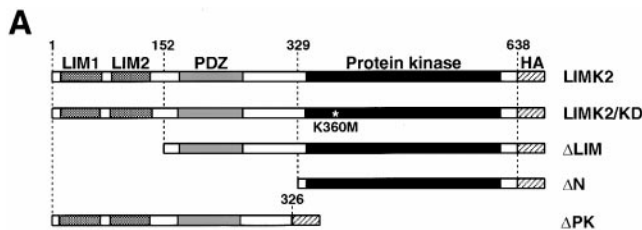


Figure 7. Protein kinase activities of LIMK2 and its mutants. **A**, Schematic structure of LIMK2 mutants. Numbers indicate amino acid residues of the NH₂ and COOH termini. Position of point mutations are indicated by an asterisk. **B**, Cofilin phosphorylation by LIMK2 and its mutants. COS-7 cells were transfected with expression vectors for HA-tagged LIMK2 and its mutants. After transient expression, protein kinase activity was measured as described for Fig. 1. Top, autoradiograph of the phosphorylated cofilin; middle, corresponding immunoblot analysis of the immunoprecipitated proteins; bottom, substrate phosphorylation was estimated, using an image analyzer (model BAS-2000; Fuji). Protein kinase activity of wild-type LIMK2 was taken as 1.0. Each value represents the means ± SE for experiments done in triplicate. The arrowheads indicate autophosphorylated LIMK2 and ΔLIM, respectively.

zation (Fig. 8). Expression of ΔLIM induced dramatic formation of stress fibers and an abnormal accumulation of the large F-actin clumps (Fig. 8, c and d), compared with findings seen with wild-type LIMK2 (Fig. 8, a and b). On the other hand, expression of ΔN and ΔPK (deletion of kinase domain) mutants was without effect regarding actin cytoskeletal changes (Fig. 8 e, f and g, h). Thus, LIMK2-induced stress fiber formation requires both PDZ domain and kinase activity. Similarly, in ΔN or ΔPK-expressed cells, we observed no alterations in vinculin-containing focal adhesion complexes (data not shown). It is noteworthy that the potential to induce stress fiber formation in mu-

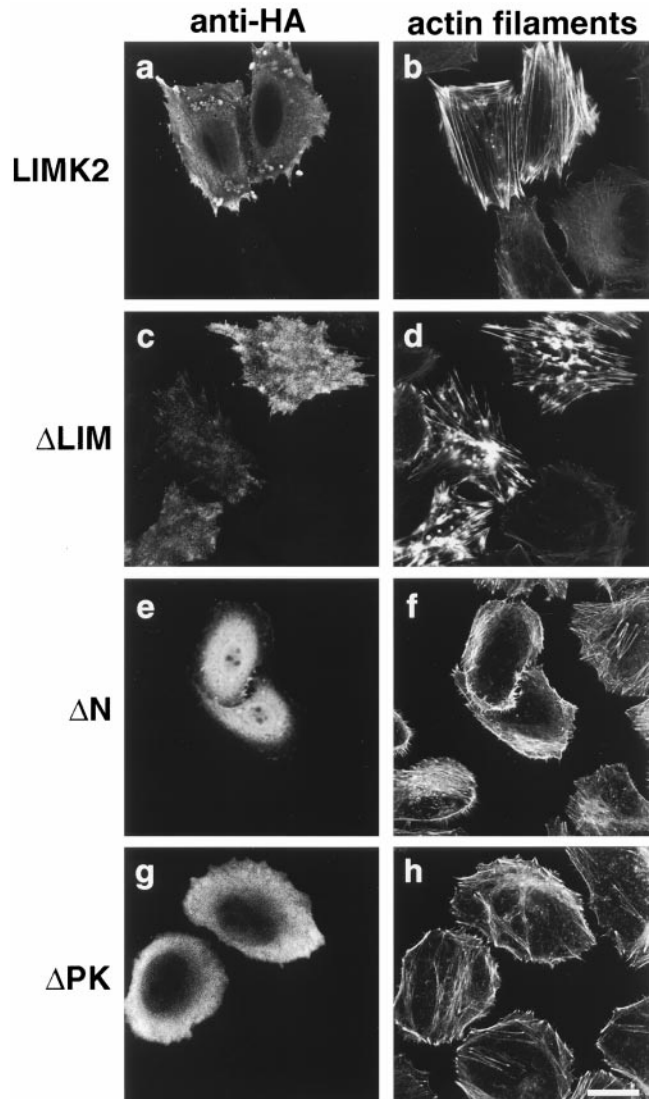


Figure 8. Effects of LIMK2 mutants on actin reorganization. Serum-starved HeLa cells were transfected with DNA constructs encoding HA-tagged wild-type LIMK2 (a and b), ΔLIM (c and d), ΔN (e and f), and ΔPK (g and h). Cells were fixed 24 h after DNA transfection and double stained with anti-HA antibody (a, c, e, and g) and phalloidin (b, d, f, and h). Confocal images were obtained as described in Materials and Methods. The results shown are representative of three independent experiments. Bar, 20 μm.

tant LIMK2 correlates with their protein kinase activity for cofilin. Perhaps deletion of LIM domains in LIMK2 may convert it to a constitutive active form, the results being increased cofilin phosphorylation and remarkable stress fiber formation. In contrast, deletion of the NH₂-terminal region containing the PDZ domain or protein kinase domain results in marked reduction or loss of cofilin phosphorylation, thus the mutant LIMK2 had no effect on actin filament dynamics. Furthermore, these domains seem to be involved in the subcellular localization of LIMK2. Immunofluorescence analysis revealed that the wild-type LIMK2 and ΔLIM mutant mainly localized in the cytoplasm and in peripheral membranous areas in

transfected cells (Fig. 8, a and c). In contrast, the ΔN mutant was predominantly localized in the nucleus, not in peripheral membranous areas (Fig. 8 e). The PDZ domain may define the subcellular localization of LIMK2 and, if so, may play an important role in reorganization of the actin cytoskeleton.

Finally, we wanted to know if these domains in LIMK2 could function as effector domains in the Cdc42- and Rho-mediated signal transduction toward cofilin phosphorylation (Fig. 9). Consistent with data in Fig. 1, the wild-type LIMK2 activity was enhanced by about twofold by coexpression of the active form of Cdc42 (Cdc42V12), however, the dominant-negative form of Cdc42 (Cdc42N17) did not modulate the wild-type LIMK2 activity (Fig. 9 A). Similar to findings with the wild-type LIMK2, the kinase activity of ΔLIM and ΔN mutants was increased by about twofold by coexpression of Cdc42V12, and was not significantly changed by the coexpression of Cdc42N17. Similar results were observed when the wild-type and truncated mutant LIMK2 was coexpressed with the active form of Rho (RhoV14) or the dominant-negative form of RhoN19. Cofilin phosphorylation of ΔLIM and ΔN mutants was increased by about threefold by the coexpression of RhoV14, while coexpression of RhoN19 did not affect the kinase activity (Fig. 9 B). Therefore, the COOH-terminal half region containing the protein kinase domain is likely to be the effector domain which confers responsiveness in LIMK2 to Cdc42- and Rho-mediated signaling pathways.

Discussion

We have provided evidence herein that LIMK2 phosphor-

ylates cofilin and induces formation of actin stress fibers and focal adhesion complexes. The LIMK2-induced cofilin phosphorylation may have inhibited its activity to depolymerize actin filaments *in vivo*, thereby leading to stabilization of actin filaments and assembly of focal adhesion complexes. It should be emphasized that, distinct from LIMK1, LIMK2 activity is regulated by Rho and Cdc42, but not Rac, and LIMK2 has a definitive role both in Rho-induced stress fiber and Cdc42-induced filopodia formation, as a novel downstream effector of Rho and Cdc42. In contrast, consistent with previous reports (Arber et al., 1998; Yang et al., 1998b), LIMK1 also phosphorylates cofilin downstream of Rac and Cdc42, while activation of LIMK1 by Cdc42 is mediated by Rac activation. The LIMK1/KD specifically inhibits the Rac-induced lamellipodia and focal adhesion complex formations, but not Cdc42- and Rho-induced cytoskeletal changes. These results indicate that LIMK1 and LIMK2 function under control of distinct Rho subfamily GTPases and are regulators in the Rho subfamily GTPases-induced rapid actin cytoskeletal reorganization. Nevertheless, expression of cofilin-S3A did not reverse the actin reorganization induced by Rho subfamily GTPases. The result suggests the involvement of phosphorylation-independent regulation of cofilin and the presence of other target(s) of LIMKs in the Rho subfamily GTPases-induced actin reorganization.

Although mechanisms governing focal complex formation are not fully understood, previous studies did suggest distinct pathways by which Rho may affect focal complex formation. Rho stimulates the activity of phosphatidylinositol 4-phosphate 5-kinase (PIP5K), an enzyme that produces phosphatidylinositol 4,5-bisphosphate (PIP2; Chong et al., 1994). PIP2 induces a conformational change

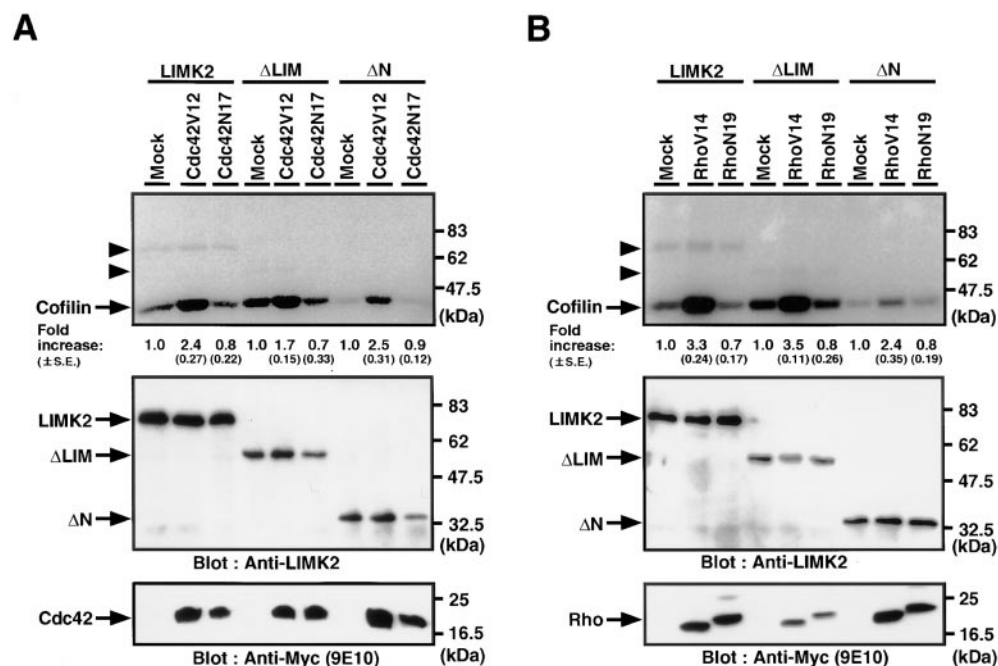


Figure 9. Regulation of protein kinase activity of LIMK2 and its mutants by Rho and Cdc42. **A**, Regulation of LIMK2 and mutant LIMK2 activities by Cdc42. COS-7 cells were coexpressed with HA-tagged wild-type LIMK2, ΔLIM , or ΔN , and empty vector (Mock), Myc-tagged Cdc42V12, or Cdc42N17. **B**, Regulation of LIMK2 and mutant LIMK2 activities by Rho. COS-7 cells were coexpressed with HA-tagged wild-type LIMK2, ΔLIM , or ΔN , and empty vector (Mock), Myc-tagged RhoV14, or RhoN19. After transient expression, LIMK2 or mutant LIMK2 kinase activity was measured as described for Fig. 1. The initial extracts (50 μ g) were also subjected to anti-Myc immunoblot. The arrowheads indicate autophosphorylated LIMK2 and ΔLIM . Cofilin phosphorylation was estimated, using an image analyzer (model BAS-2000; Fuji), and the amount of cofilin phosphorylation by LIMK2 expressed with Mock was respectively taken as 1.0. The results shown are representative of three independent experiments. Each value represents the mean \pm SE of three independent experiments.

phorylated LIMK2 and ΔLIM . Cofilin phosphorylation was estimated, using an image analyzer (model BAS-2000; Fuji), and the amount of cofilin phosphorylation by LIMK2 expressed with Mock was respectively taken as 1.0. The results shown are representative of three independent experiments. Each value represents the mean \pm SE of three independent experiments.

in vinculin, which allows for interaction with talin, thereby suggesting a role for PIP₂ in assembly of focal adhesions (Gilmore and Burridge, 1996). On the other hand, Rho also activates Rho-kinase/ROCK, which plays an important role in assembly of focal adhesions (Amano et al., 1997; Chihara et al., 1997). We propose that LIMKs are also, at least in part, involved in the Rho subfamily GTPases-induced focal complex formation, since expression of the LIMKs/KD significantly inhibited the activated Rho GTPases-induced focal adhesion complexes. Nevertheless, since our experiments were done using cells overexpressing LIMKs, we cannot exclude the possibility that overexpressed LIMKs might be sequestering other protein(s) involved in cytoskeletal rearrangements driven by Rho subfamily GTPases. The knockout of LIMKs in cells and tissues may lead to elucidation of roles of LIMKs in normal biological and physiological processes, including cytoskeletal regulation.

LIMKs play a role in Rho subfamilies-induced cytoskeletal changes and LIMKs-induced actin cytoskeletal reorganization seems to be, at least in part, mediated by inactivation of the depolymerizing activity of cofilin, since the overexpression of cofilin-S3A inhibits LIMK2-induced actin stress fibers and focal adhesion complexes. Likewise, the LIMKs/KD inhibit Rho subfamilies-induced cytoskeletal changes. However, dominant expression of cofilin-S3A had no effect on the Rho-induced stress fiber, Rac-induced lamellipodia, and Cdc42-induced filopodia formations in our system. These results do raise the following possibilities. First, cofilin functions may be regulated by mechanisms distinct from its phosphorylation downstream of Rho subfamily GTPases. Functions of cofilin are regulated by other factors such as pH change, PIP₂, and the capping mechanism of actin filaments, as well as protein phosphorylation (Bamburg et al., 1999). Secondly, LIMKs also are likely to be involved in actin cytoskeletal reorganization by regulating molecule(s) other than cofilin, under Rho subfamily GTPases. This notion is supported by the observation that LIMK2/KD inhibits Rho-induced stress fiber formation, but does allow for accumulation of actin filaments. In addition to cofilin-mediated actin depolymerization, LIMK2 may regulate other target molecule(s) involved in bundling and/or cross-linking of actin filaments. This notion remains to be addressed.

Our results raise the question as to how LIMK1 and LIMK2 are specifically activated downstream of Rho subfamily GTPases. Most effector molecules of Rho subfamily GTPases have specific binding regions, i.e., Cdc42/Rac interactive binding domain (Burbelo et al., 1995) or Rho-binding domain (Reid et al., 1996; Fujisawa et al., 1998). However, neither LIMK1 nor LIMK2 contain these characteristic domains and we found that LIMK1 and LIMK2 do not directly associate with Rho, Rac, and Cdc42 (data not shown). LIMKs may possibly be activated indirectly by Rho subfamily GTPases. One likely mechanism is the protein phosphorylation of LIMKs. Maekawa et al. (1999) reported that LIMK1 and LIMK2 are phosphorylated and activated by Rho-kinase/ROCK, Rho-dependent protein kinase (Ishizaki et al., 1996; Leung et al., 1996; Matsui et al., 1996). However, their results seem to contradict previous data (Arber et al., 1998; Yang et al., 1998b), as do our present findings that LIMK1 is regulated by Rac, while

LIMK2 is regulated by Rho and Cdc42. Edwards et al. (1999) reported that Pak1 (p21-activated kinase) directly binds to LIMK1, and this association is enhanced by activated Rac or Cdc42. Moreover, Pak1 phosphorylates a critical threonine residue (T508) present in the activation loop of LIMK1, which results in activation of LIMK1 (Edwards and Gill, 1999; Edwards et al., 1999). In addition to Rho-kinase/ROCK and Pak, Rho subfamily GTPases activate other protein kinases, including citron kinase (Madaule et al., 1998), PRK2 (Vincent and Settleman, 1997), and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK; Leung et al., 1998). Whether LIMKs are activated by these potential protein kinases and how LIMK1 and LIMK2 are specifically regulated under Rho subfamily GTPases remain to be addressed.

Although the LIM domain was initially noted in homeobox-containing transcription factors (Way and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990), it also was found in a variety of proteins (Sanchez-Garcia and Rabbitts, 1994). Some LIM proteins are comprised almost exclusively of LIM domains, whereas other LIM proteins have LIM domains linked to other functional domains, including homeobox, protein kinase, or other domains. In LIM homeodomain proteins, the LIM domain interacts with the homeodomain and inhibits DNA binding activity (Sanchez-Garcia et al., 1993; Xue et al., 1993; Taira et al., 1994), while this inhibitory effect is competitively released by LIM domain inactivator proteins. On the other hand, the LIM domain in LIMK2 functions at least as an autoinhibitory domain, since deletion of the LIM domain resulted in marked enhancement both in cofilin phosphorylation and concomitant stress fiber formation. Inconsistent with the LIM domain in LIMK1 that may be essential for Rac-dependent regulation (Arber et al., 1998), the LIM domain in LIMK2 is not responsible for Rho- and Cdc42-dependent regulatory functions, because deletion of the LIM domain or the NH₂-terminal half region containing LIM and PDZ domains did not affect activation of LIMK2 by Rho and Cdc42. Therefore, we propose that the COOH-terminal regions containing the protein kinase domain are involved in Rho- and Cdc42-dependent regulation of LIMK2 activity.

PDZ domains are found in diverse membrane-associated proteins, including members of the MAGUK family of guanylate kinase homologues, several protein phosphatases and kinases, and several dystrophin-associated proteins (Ponting et al., 1997). The PDZ domain appears to be a targeting signal to be localized in specialized sites at cytoplasmic surfaces, which suggests participation in cellular junction formation, receptor or channel clustering, and intracellular signaling events. We found that the PDZ domain of LIMK2 appears to be important for both protein kinase activity and for the localization of LIMK2 specialized submembranous areas, since the deletion mutant Δ N, but not Δ LIM, showed decreased protein kinase activity and aberrant subcellular localization in the nucleus, but not in peripheral membranous areas. On the other hand, the PDZ domain in LIMK1 contains the nuclear export signal-like sequence and deletion of PDZ resulted in nuclear localization (Yang et al., 1998a). Thus, a similar aberrant subcellular localization caused by deletion of the PDZ domain in LIMK1 and LIMK2 suggests a functional

similarity for the PDZ domain. However, the PDZ domain of LIMK2 has no typical nuclear export signal. We speculate that subcellular localization of LIMK2 may be determined by other mechanisms, such as interaction with scaffolding, anchoring, and adapter proteins through LIM and PDZ domains.

LIMK1 is preferentially, but not exclusively expressed in the brain, whereas LIMK2 is ubiquitously expressed in a variety of tissues (Mizuno et al., 1994; Bernard et al., 1994; Nunoue et al., 1995; Okano et al., 1995; Ikebe et al., 1997; Koshimizu et al., 1997). During development, LIMK1 and LIMK2 are preferentially expressed and are mostly colocalized in central nervous systems (CNS) during mid-to-late gestation, however, differential gene expression is observed in some nuclei during CNS development (Mori et al., 1997). Together with closely related structural features of LIMK1 and LIMK2, LIMKs are likely to share similar, but distinct, biological functions with a particular function for LIMK1 in postnatal CNS. Indeed, LIMK1 hemizyosity has been implicated in the pathogenesis of the visuospatial constructive cognitive defect of Williams syndrome (Frangiskakis et al., 1996). We find that function and activities of LIMKs toward actin cytoskeletal reorganization are regulated in a distinct manner by Rho subfamily small GTPases. We predict that LIMKs have distinct biological and physiological functions; in some cases they may be counteracting whereas in the others they may be cooperative. Ongoing gene disruption analysis of LIMK1 and LIMK2 in cells and in mice should reveal biological functions, as well as functional distinctions between LIMK1 and LIMK2.

In summary, we conclude that LIMK2 plays the role of a novel downstream target of Rho and Cdc42 in actin cytoskeletal reorganization, and is a regulator for cofilin-mediated actin depolymerization. This seems to be the first identification of LIMK2 as a regulator of actin depolymerization, at least in part, via cofilin phosphorylation downstream of Rho and Cdc42. Coupling of Rho subfamily GTPases and actin dynamics, both involved in diverse cellular functions, has been given new interest and direction.

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References

- Abe, H., T. Obinata, L.S. Minamide, and J.R. Bamberg. 1996. *Xenopus laevis* actin-depolymerizing factor/cofilin: a phosphorylation-regulated protein essential for development. *J. Cell Biol.* 132:871–885.
- Agnew, B.J., L.S. Minamide, and J.R. Bamberg. 1995. Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site. *J. Biol. Chem.* 270:17582–17587.
- Allen, W.E., G.E. Jones, J.W. Pollard, and A.J. Ridley. 1997. Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J. Cell Sci.* 110:707–720.
- Amano, M., K. Chihara, K. Kimura, Y. Fukata, N. Nakamura, Y. Matsuura, and K. Kaibuchi. 1997. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science.* 275:1308–1311.
- Arber, S., F.A. Barbayannis, H. Hanser, C. Schneider, C.A. Stanyon, O. Bernard, and P. Caroni. 1998. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature.* 393:805–809.
- Ayscough, K.R. 1998. In vivo functions of actin-binding proteins. *Curr. Opin. Cell Biol.* 10:102–111.
- Bamburg, J.R., A. McGough, and S. Ono. 1999. Putting a new twist on actin: ADF/cofilins modulate actin dynamics. *Trends Cell Biol.* 9:364–370.
- Bernard, O., S. Ganiatsas, G. Kannourakis, and R. Dringen. 1994. Kiz-1, a protein with LIM zinc finger and kinase domains, is expressed mainly in neurons. *Cell Growth Differ.* 5:1159–1171.
- Burbelo, P.D., D. Drechsel, and A. Hall. 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem.* 270:29071–29074.
- Carlier, M.-F., and D. Pantaloni. 1997. Control of actin dynamics in cell motility. *J. Mol. Biol.* 269:459–467.
- Chihara, K., M. Amano, N. Nakamura, T. Yano, M. Shibata, T. Tokui, H. Ichikawa, R. Ikebe, and K. Kaibuchi. 1997. Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *J. Biol. Chem.* 272:25121–25127.
- Chong, L.D., A. Traynor-Kaplan, G. Bohoch, and M.A. Schwartz. 1994. The small GTP-binding protein rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell.* 79:507–513.
- Edwards, D.C., and G.N. Gill. 1999. Structural features of LIM kinase that control effects on the actin cytoskeleton. *J. Biol. Chem.* 274:11352–11361.
- Edwards, D.C., L.C. Sanders, G.M. Bokoch, and G.N. Gill. 1999. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signaling to actin cytoskeletal dynamics. *Nat. Cell Biol.* 1:253–259.
- Frangiskakis, J.M., A.K. Ewart, C.A. Morris, C.B. Mervis, J. Bertrand, B.F. Robinson, B.P. Klein, G.J. Ensing, L.A. Everett, E.D. Green, et al. 1996. LIM-kinase1 hemizyosity implicated in impaired visuospatial constructive cognition. *Cell.* 86:59–69.
- Freyd, G., S.K. Kim, and H.R. Horvitz. 1990. Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene lin-11. *Nature.* 344:876–879.
- Fujisawa, K., P. Madaule, T. Ishizaki, G. Watanabe, H. Bito, Y. Saito, A. Hall, and S. Narumiya. 1998. Different regions of Rho determine Rho-selective binding of different classes of Rho target molecules. *J. Biol. Chem.* 273:18943–18949.
- Gilmore, A.P., and K. Burridge. 1996. Regulation of vinculin binding to talin and actin by phosphatidylinositol-4-5-bisphosphate. *Nature.* 381:531–535.
- Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science.* 279:509–514.
- Ikebe, C., K. Ohashi, T. Fujimori, O. Bernard, T. Noda, E.J. Robertson, and K. Mizuno. 1997. Mouse LIM-kinase 2 gene: cDNA cloning, genomic organization, and tissue-specific expression of two alternatively initiated transcripts. *Genomics.* 46:504–508.
- Ishizaki, T., M. Maekawa, K. Fujisawa, K. Okawa, A. Iwamatsu, A. Fujita, N. Watanabe, Y. Saito, A. Kakizuka, N. Morii, et al. 1996. The small GTP-binding protein Rho binds to and activates a 160 kDa Sea/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:1885–1893.
- Karlsson, O., S. Thor, T. Norberg, H. Ohlsson, and T. Edlund. 1990. Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature.* 344:879–882.
- Komuro, R., T. Sasaki, K. Takaishi, S. Orita, and Y. Takai. 1996. Involvement of Rho and Rac small G proteins and Rho GDI in Ca²⁺-dependent exocytosis from PC12 cells. *Genes to Cells.* 1:943–951.
- Koshimizu, U., H. Takahashi, M.C. Yoshida, and T. Nakamura. 1997. cDNA cloning, genomic organization, and chromosomal localization of the mouse LIM motif-containing kinase gene, *Limk2*. *Biochem. Biophys. Res. Commun.* 241:243–250.
- Kozma, R., S. Ahmed, A. Best, and L. Lim. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell Biol.* 15:1942–1952.
- Lappalainen, P., and D.G. Drubin. 1997. Cofilin promotes rapid actin filament turnover in vivo. *Nature.* 388:78–82.
- Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell.* 84:359–369.
- Leung, T., X.-Q. Chen, E. Manser, and L. Lim. 1996. The p160 RhoA-binding kinase ROKa is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell Biol.* 16:5313–5327.
- Leung, T., X.-Q. Chen, I. Tan, E. Manser, and L. Lim. 1998. Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol. Cell Biol.* 18:130–140.
- Madaule, P., M. Eda, N. Watanabe, K. Fujisawa, T. Matsuoka, H. Bito, T. Ishizaki, and S. Narumiya. 1998. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature.* 394:491–494.
- Maekawa, M., T. Ishizaki, S. Boku, N. Watanabe, A. Fujita, A. Iwamatsu, T. Obinata, K. Ohashi, K. Mizuno, and S. Narumiya. 1999. Signaling from Rho to the actin cytoskeleton through protein kinase ROCK and LIM-kinase. *Science.* 285:895–898.
- Matsui, T., M. Amano, T. Yamamoto, K. Chihara, M. Nakafuku, M. Ito, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP-binding protein Rho. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2208–2216.
- Matsui, T., M. Maeda, Y. Doi, S. Yonemura, M. Amano, K. Kaibuchi, S. Tsukita, and S. Tsukita. 1998. Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J. Cell Biol.* 140:647–657.
- Mitchison, T.J., and L.P. Cramer. 1996. Actin-based cell motility and cell loco-

- motion. *Cell* 84:371–379.
- Mizuno, K., I. Okano, K. Ohashi, K. Nunoue, K. Kuma, T. Miyata, and T. Nakamura. 1994. Identification of a human cDNA encoding a novel protein kinase with two repeats of the LIM/double zinc finger motif. *Oncogene* 9:1605–1612.
- Moon, A., and D.G. Drubin. 1995. The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics. *Mol. Biol. Cell* 6:1423–1431.
- Mori, T., I. Okano, K. Mizuno, M. Tohyama, and A. Wanaka. 1997. Comparison of tissue distribution of two novel serine/threonine kinase genes containing the LIM motif (LIMK-1 and LIMK-2) in the developing rat. *Brain Res. Mol. Brain Res.* 45:247–254.
- Moriyama, K., K. Iida, and I. Yahara. 1996. Phosphorylation of Ser-3 of cofilin regulates its essential function on actin. *Genes to Cells* 1:73–86.
- Nobes, C.D., and A. Hall. 1995. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81:53–62.
- Nunoue, K., K. Ohashi, I. Okano, and K. Mizuno. 1995. LIMK-1 and LIMK-2, two members of a LIM motif containing protein kinase family. *Oncogene* 11:701–710.
- Okano, I., J. Hiraoka, H. Otera, K. Nunoue, K. Ohashi, S. Iwashita, M. Hirai, and K. Mizuno. 1995. Identification and characterization of a novel family of serine/threonine kinases containing two N-terminal LIM motifs. *J. Biol. Chem.* 270:31321–31330.
- Ponting, C.P., C. Phillips, K.E. Davies, and D.J. Blake. 1997. PDZ domains: targeting signaling molecules to sub-membranous sites. *Bioessays* 19:469–479.
- Reid, T., T. Furiyashiki, T. Ishizaki, G. Watanabe, N. Watanabe, K. Fujisawa, N. Morii, P. Madaule, and S. Narumiya. 1996. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rho-philin in the rho-binding domain. *J. Biol. Chem.* 271:13556–13560.
- Ridley, A.J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70:389–399.
- Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 70:401–410.
- Sanchez-Garcia, I., and T.H. Rabbitts. 1994. The LIM domain: a new structural motif found in zinc finger-like proteins. *Trends Genet.* 10:315–320.
- Sanchez-Garcia, I., H. Osada, A. Forster, and T.H. Rabbitts. 1993. The cysteine-rich LIM domains inhibit DNA binding by the associated homeodomain in Isl-1. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4243–4250.
- Taira, M., H. Otani, J.P. Saint-Jeannet, and I.B. Dawid. 1994. Role of the LIM class homeodomain protein Xlim-1 in neural and muscle induction by the Spemann organizer in *Xenopus*. *Nature* 372:677–679.
- Takahashi, H., U. Koshimizu, and T. Nakamura. 1998. A novel transcript encoding truncated LIM kinase 2 is specifically expressed in male germ cells undergoing meiosis. *Biochem. Biophys. Res. Commun.* 249:138–145.
- Takai, Y., T. Sasaki, K. Tanaka, and H. Nakanishi. 1995. Rho as a regulator of the cytoskeleton. *Trends Biochem. Sci.* 20:227–231.
- Theriot, J.A. 1997. Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton. *J. Cell Biol.* 136:1165–1168.
- Van Aelst, L., and C. D'Souza-Schorey. 1997. Rho GTPases and signaling networks. *Genes Dev.* 11:2295–2322.
- Vincent, S., and J. Settleman. 1997. The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. *Mol. Cell. Biol.* 17:2247–2256.
- Way, J.C., and M. Chalfie. 1988. *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. *Cell* 54:5–16.
- Xue, D., Y. Tu, and M. Chalfie. 1993. Cooperative interactions between the *Caenorhabditis elegans* homeoproteins UNC-86 and MEC-3. *Science* 261:1324–1328.
- Yang, N., O. Higuchi, and K. Mizuno. 1998a. Cytoplasmic localization of LIM-kinase 1 is directed by a short sequence within the PDZ domain. *Exp. Cell Res.* 241:242–252.
- Yang, N., O. Higuchi, K. Ohashi, K. Nagata, A. Wada, K. Kangawa, E. Nishida, and K. Mizuno. 1998b. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 393:809–812.
- Yonezawa, N., E. Nishida, K. Iida, I. Yahara, and H. Sakai. 1990. Inhibition of the interactions of cofilin, dectin, and deoxyribonuclease I with actin by phosphoinositides. *J. Biol. Chem.* 265:8382–8386.