Specific Activation of LIM kinase 2 via Phosphorylation of Threonine 505 by ROCK, a Rho-dependent Protein Kinase*

Received for publication, August 4, 2000, and in revised form, September 18, 2000
Published, JBC Papers in Press, October 3, 2000, DOI 10.1074/jbc.M007074200

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LIM-kinase 1 (LIMK1) and LIM-kinase 2 (LIMK2) regulate actin cytoskeletal reorganization via cofilin phosphorylation downstream of distinct Rho family GTPases. We report our findings that ROCK, a downstream protein kinase of Rho, specifically activates LIMK2 but not LIMK1 downstream of RhoA. LIMK1 and LIMK2 activities toward cofilin phosphorylation were stimulated by co-expression with the active form of ROCK (ROCKΔ3), whereas full-length ROCK selectively activates LIMK2 but not LIMK1. Activation of LIMK2 by RhoA was inhibited by Y-27632, a specific inhibitor of ROCK, but Rac1-mediated activation of LIMK1 was not. ROCK directly phosphorylated the threonine 505 residue within the activation segment of LIMK2 and markedly stimulated LIMK2 activity. A LIMK2 mutant with replacement of threonine 505 by valine abolished LIMK2 activities for cofilin phosphorylation and actin cytoskeletal changes, whereas replacement by glutamate enhanced the protein kinase activity and stress fiber formation by LIMK2. These results indicate that ROCK directly phosphorylates threonine 505 and activates LIMK2 downstream of RhoA and that this phosphorylation is essential for LIMK2 to induce actin cytoskeletal reorganization. Together with the finding that LIMK1 is regulated by Pak1, LIMK1 and LIMK2 are regulated by different protein kinases downstream of different Rho family GTPases.

Cell division, motility, and shape determination depend on reorganization of the actin cytoskeleton. Actin cytoskeletal dynamics is precisely regulated by a variety of actin-binding proteins involved in polymerization/dem polymerization, capping, and bundling of actin filaments (1, 2). Extensive remodeling of the actin cytoskeleton with exposure to extracellular signals is regulated by Rho family GTPases (including Rho, Rac, and Cdc42), and each member participates in the specific regulation of the actin cytoskeleton and various cell adhesion-related events (3–6). Several direct or indirect downstream effectors of Rho family GTPases have been identified, but signal transduction pathways by which Rho family GTPases activate actin cytoskeleton. Among them, ROCK, an isoform of Rho-kinase, plays a key role in formation of actin stress fibers downstream of Rho (21–26). Previous reports showed that the constitutive active form of ROCK activates both LIMK1 and LIMK2 (27) and that LIMK1 is activated by wild-type ROCK (28). However, these reports contradict a previous notion that LIMK1 is specifically activated by Rac but not by Rho (18–20). In the present study, we asked if ROCK could specifically activate LIMK2 downstream of RhoA. We found that signal transduction pathways wherein ROCK directly phosphorylates and activates LIMK2, but not LIMK1 downstream of RhoA resulted in phosphorylation and inactivation of the actin-depolymerizing factor cofilin.

EXPERIMENTAL PROCEDURES

Materials—Fluorescein isothiocyanate-conjugated anti-mouse IgG, anti-hemagglutinin (HA) monoclonal antibody (12CA5), and anti-Myc monoclonal antibody (9E10) were purchased from Roche Molecular Biochemicals. Anti-HA (Y-11), anti-Myc (A-14) polyclonal antibody and rhodamine-conjugated phallolidin were, respectively, purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Molecular Probes, Inc. (Eugene, OR). Anti-LIMK2 polyclonal antibody was generated as described elsewhere (29). LipofectAMINE and Opti-MEM were purchased from Life Technologies, Inc. Y-27632 was generously provided by WelFide Co. (Osaka, Japan).

Construction of Expression Plasmids and Preparation of Reconstituted Protein—The expression plasmids for Rho family GTPases (pEF-BOS-myc), HA-tagged LIMK1, LIMK2 and its mutants, and glutathione S-transferase (GST)-fused cofilin were constructed as described (20). Expression plasmids for Myc-tagged ROCK and its mutants were kindly provided by Dr. S. Narumiya (Kyoto University, Kyoto, Japan). The cDNA for T494V and T494E mutants of LIMK2 were constructed to

t/or cofilin family play a definitive role in depolymerization of actin filaments (7, 8) and, hence, are potential downstream effectors of signaling cascades that evoke actin cytoskeletal reorganization. A mechanism involved in regulating cofilin function is phosphorylation of an N-terminal serine residue of cofilin, through which its F-actin-depolymerizing activity is inactivated (9, 10). Studies showed that LIM-kinase (LIMK),1 LIM domain-containing serine/threonine/protein-tyrosine kinase (11–17), phosphorylates cofilin downstream of Rho family GTPases (18–20). We report that two types of LIMKs, LIMK1 and LIMK2, are regulated in a distinct manner downstream of Rho family GTPases. LIMK1 is specifically activated downstream of Rac1 and Cdc42 but not RhoA, whereas LIMK2 is specifically activated downstream of RhoA and Cdc42 but not Rac1 (20). LIMKs may be indirectly activated by Rho family GTPases, because LIMKs are not a direct target of these GTPases.

Several target proteins of Rho are involved in regulating the actin cytoskeleton. Among them, ROCK, an isoform of Rho-kinase, plays a key role in formation of actin stress fibers downstream of Rho (21–26). Previous reports showed that the constitutive active form of ROCK activates both LIMK1 and LIMK2 (27) and that LIMK1 is activated by wild-type ROCK (28). However, these reports contradict a previous notion that LIMK1 is specifically activated by Rac but not by Rho (18–20). In the present study, we asked if ROCK could specifically activate LIMK2 downstream of RhoA. We found that signal transduction pathways wherein ROCK directly phosphorylates and activates LIMK2, but not LIMK1 downstream of RhoA resulted in phosphorylation and inactivation of the actin-depolymerizing factor cofilin.

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introduce substitution of Thr-494 by Val and Glu residues, respectively, using a site-directed mutagenesis kit (CLONTECH, Palo Alto, CA). The plasmids for T505V, T505E, and T494V/T505V mutants of LIMK2 were generated in a similar manner. To generate the plasmid encoding for the GST-fused protein kinase domain of the kinase-dead LIMK2 (GST-PK), the 494V/T505V mutants, the cDNA fragment of the kinase-dead LIMK2 was amplified by polymerase chain reaction using a set of the following primers: forward, 5’-GCGGATCCCTCTGGACCTGATCAC-3’, and the reverse, 5’-GCGGATCCCTAGGGTGGCAGGTCC-3’. The polymerase chain reaction product digested with BamHI was ligated to the BamHI-digested pGEX-6P-2 vector (Amersham Pharmacia Biotech). The authenticity of these expression plasmids was confirmed by nucleotide sequence analysis. The plasmids coding for GST-fused cofilin, GST-PK, and its mutants were transformed into Escherichia coli BL-21. Expression and purification of recombinant protein were done according to the manufacturer’s protocol.

Transfection—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. HeLa cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and non-essential amino acids. Subconfluent COS-7 cells were trypsinized and resuspended in phosphate-buffered saline (PBS), and 10⁶ cells were transfected with 10 µg of plasmid DNA by electroporation using a Gene Pulser (Bio-Rad) according to the manufacturer’s instructions. Cells were cultured for 36 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

HeLa cells were plated on a glass coverslip at a density of 6 x 10⁶/cm², cultured for 12 h, then further cultured for 16 h in serum-free minimal essential medium. The cells were transfected in Opti-MEM containing 1 µg of plasmid DNA complexed with LipofectAMINE. After a 2-h incubation, the medium was replaced with serum-free minimal essential medium, and the cells were further cultured for 22 h, then fixed and stained.

Immunoprecipitation, Immunoblot Analysis, and Protein Kinase Assay—COS-7 cells were transiently transfected with expression plasmid, as described above, then cultured for 36 h. The cells were lysed in 1 ml of lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, 25 mM β-glycerophosphate, 10 mM NaF, 1 mM Na₂VO₄, 1% Triton X-100, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin and aprotinin, and incubated on ice for 30 min. After centrifugation, the supernatant was preadsorbed with 15 µl of protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C and centrifuged to remove protein G-Sepharose, and the supernatant was incubated for 3 h at 4 °C with anti-HA or anti-Myc antibody and 5 µl of protein G-Sepharose. Protein G-Sepharose beads were washed three times with lysis buffer and dissolved in the sample buffer for SDS-polyacrylamide gel electrophoresis. The immunoprecipitates and cell lysates were, separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) and probed with anti-HA, anti-Myc, or anti-LIMK2 antibody. Proteins reacting with these antibodies were detected using ECL enhanced chemiluminescence (Amer-sham Pharmacia Biotech). The authenticity of these expression plasmids was confirmed by nucleotide sequence analysis. The plasmids coding for GST-fused cofilin, GST-PK, and its mutants were transformed into Escherichia coli BL-21. Expression and purification of recombinant LIMK1 and LIMK2 were done as described (30). The region of the polyacrylamide gel containing the radioactive protein was excised and eluted, protein from the gel was incubated with 6 N HCl at 110 °C for 1 h, and hydrolysates were separated on a thin-layer cellulose plate using of the Hunter thin-layer electrophoresis system (HTLE-7000; CBS Scientific, Del Mar, CA). The ³²P-labeled phosphoamino acids were detected by autoradiography, standard, and compared with the ninhydrin-stained phosphoamino acid standards.

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 for 1 h and subsequently with fluorescein isothiocyanate-conjugated anti-mouse IgG and rhodamine-conjugated phalloidin for 1 h. The cells were then washed three times with PBS, mounted on glass slides, and analyzed with use of a LSM 410 confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany).

RESULTS

Specific Activation of LIMK2 by ROCK in the Downstream of Rho—To determine if LIMKs could be activated by ROCK, wild type and mutant types of this protein kinase were, respectively, co-expressed with HA-tagged LIMK1 or LIMK2 in COS-7 cells, then we measured LIMKs activity using GST-fused cofilin as substrate (Fig. 1). ROCK-Δ3 contains the protein kinase domain and half of the coiled-coil domain, which served as the constitutively active form of ROCK (24). LIMK1 and LIMK2...
activities toward cofilin phosphorylation in cells co-expressing ROCK-Δ3 were, respectively, 5.3-fold and 7.3-fold higher than those seen in control cells expressing LIMK1 or LIMK2 alone (Fig. 1A). Likewise, autophosphorylation of LIMK1 and LIMK2 was also slightly enhanced by co-expression with ROCK-Δ3 (Fig. 1A, arrows). Importantly, however, when LIMK1 or LIMK2 was co-expressed with wild-type ROCK, LIMK2 activity toward cofilin phosphorylation was enhanced 2.8-fold, whereas LIMK1 activity was not stimulated (Fig. 1B). Stimulatory effects on LIMK1 and LIMK2 activities were not seen when LIMK1 or LIMK2 was co-expressed with the protein kinase-deficient form of ROCK (KDIA) (Fig. 1A). Similar results were obtained when these proteins were transiently expressed in HeLa and NIH3T3 cells (data not shown). The results indicate that wild-type ROCK activates LIMK2 but not LIMK1 in the cells, depending on the protein kinase activity, whereas deletion of the C-terminal half of ROCK results in a non-selective activation of LIMKs.

To further confirm the specific activation of LIMK2 by ROCK, we examined effects of Y-27632, a specific inhibitor of ROCK (31), on the activation of LIMKs by Rho family GTPases (Fig. 2). We reported that the activity of LIMK2 is regulated through the protein kinase domain of LIMK2 (20). Here, we asked if the activation of LIMK2 activity by ROCK is also through the kinase domain (Fig. 3). Consistent with data in Fig. 1A, the wild-type LIMK2 activity was enhanced 10.5-fold by the co-expression with ROCK-Δ3 (Fig. 3A). Similar to findings with the wild-type LIMK2, the kinase activity of PK mutant (which is deleted with N-terminal half containing LIM domains) was also enhanced to about 10-fold by the co-expression with ROCK-Δ3, indicating that LIMK2 through its protein kinase domain is activated by ROCK.

To further address the mechanism of ROCK-dependent LIMK2 activation, protein kinase domain of the kinase-dead LIMK2 (PK) (which did not undergo autophosphorylation in the in vitro phosphorylation assay) was prepared as the GST-fused protein, and we determined if ROCK directly phosphorylates this recombinant GST-fused PK as a substrate. As shown in Fig. 3B, ROCK-Δ3 induced phosphorylation of PK, and this phosphorylation depended on the protein kinase activity of ROCK, since the kinase-defective mutant ROCK (KDIA) did not phosphorylate PK. These results indicate that LIMK2 is directly phosphorylated and is activated by ROCK through the protein kinase domain of LIMK2. To analyze the phosphorylated amino acid residues in PK, PK was phosphorylated by ROCK-Δ3 in vitro and subjected to thin-layer electrophoresis. A phosphoamino acid analysis revealed that LIMK2 was phosphorylated by ROCK on threonine residues (Fig. 3C).

ROCK Activates LIMK2 through Phosphorylation on Threonine 505—Many protein kinases are phosphorylated on a residue(s) located in a particular segment in the kinase domain termed the activation segment, and phosphorylation of this segment is associated with the stimulation of kinase activity (32). In the corresponding activation segment of LIMK2, two threonine residues at positions 494 and 505 are conserved among species (Fig. 4A). Since sequences RKRT and KRYT surrounding the Thr-494 and Thr-505, respectively, are consistent with the consensus recognition sequence for Rho-kinase/ROCK (XKX_{ss}ST) (single letter code in which X denotes any residue) (33), we considered that these two threonine residues might be potential phosphorylation sites for LIMK2 activation by ROCK. To address this issue, we prepared mutant protein kinase domains of LIMK2 in which Thr-494 and Thr-505 were, respectively, replaced with valine residues, and we asked if ROCK could phosphorylate these mutant PKs (Fig. 4B). When these proteins were incubated with ROCK-Δ3, both
ROCK-contrast, there was a remarkable decrease in phosphorylation by ROCK. After transient expression, immunoprecipitated LIMK2 and PK were used for in vitro kinase assay using GST-cofilin as substrate (top panel) and for anti-HA immunoblot (middle panel). Cell lysates (50 μg) were also used for anti-Myc immunoblot (bottom panel). The arrow indicates autophosphorylated full-length LIMK2. The amount of cofilin phosphorylated by LIMK2 in Mock cells was taken as 1.0. Each value represents the mean ± S.E. of three independent experiments. B, phosphorylation of PK by ROCK. COS-7 cells were transfected with expression vector for Myc-tagged ROCK-Δ3 or KDIA. After transient expression, immunoprecipitated ROCK-Δ3 and KDIA were used for in vitro kinase assay using GST or GST-PK as substrate (top panel) and for anti-Myc immunoblot (middle panel). Substrates were stained by Coomassie Brilliant Blue (C.B.B.) (bottom panel). The arrow indicates autophosphorylated ROCK-Δ3. C, phosphoamino acid analysis of phosphorylated PK. The 32P-labeled GST-PK band was excised from the polyacrylamide gel prepared as in B, hydrolyzed, and analyzed by thin-layer electrophoresis. Positions of phosphorysine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr), sample origin (Ori), and free phosphate (Pi) are indicated.

To further examine the requirement for Thr-494 and Thr-505 as regulatory phosphorylation sites in LIMK2 by ROCK, we constructed the expression plasmids for mutated full-length LIMK2 in which both Thr-494 and Thr-505 were replaced with valine (LIMK2T494V). LIMK2 wild-type PK and PKT494V mutants were phosphorylated by ROCK-Δ3, depending on the protein kinase activity. In contrast, there was a remarkable decrease in phosphorylation by ROCK-Δ3 with the PKT505V mutant, indicating that Thr-505 is a major site phosphorylated by ROCK. However, since marginal phosphorylation was still seen in PKT505V, ROCK may weakly phosphorylate the kinase domain of LIMK2 in addition to Thr-505. Consistent with this notion, a double mutant of PK (in which both Thr-494 and Thr-505 were replaced with valine) was not phosphorylated by ROCK (data not shown).

To determine if ROCK regulates LIMK2 activity through direct phosphorylation of Thr-505 in vivo, we expressed ROCK-Δ3 together with LIMK2T505V or LIMK2T505E and examined the potential of ROCK-Δ3 to activate each mutant LIMK2 (Fig. 6). The kinase activity of wild-type LIMK2 was enhanced to a 9.6-fold higher level by co-expression with ROCK-Δ3. Similar results were also obtained in both LIMK2T494V and LIMK2T494E mutants co-expressing ROCK-Δ3 (data not shown). In contrast, neither LIMK2T505V nor LIMK2T505E was enhanced by co-expression with ROCK-Δ3. ROCK can thus exert its effect only if there is a phosphorylatable threonine residue at position 505 within the activation segment of LIMK2, and ROCK apparently directly regulates LIMK2 activity through the phosphorylation of Thr-505.

We reported that LIMK1 and LIMK2 function downstream of Rho family GTPases (20). In the present study, we found that the LIMK2 activity was specifically activated by ROCK downstream of RhoA and that ROCK directly phosphorylated at Thr-505 in the activation segment of LIMK2. Moreover, phosphorylation of this site was essential for actin cytoskeletal change and cofilin phosphorylation by LIMK2. On the other hand, it has been reported that LIMK1 is phosphorylated and activated by Pak1 (34). Taken together, these observations suggest that LIMK1 and LIMK2 are regulated by

**DISCUSSION**

We reported that LIMK1 and LIMK2 function downstream of distinct Rho family GTPases (20). In the present study, we found that the LIMK2 activity was specifically activated by ROCK downstream of RhoA and that ROCK directly phosphorylated at Thr-505 in the activation segment of LIMK2. Moreover, phosphorylation of this site was essential for actin cytoskeletal change and cofilin phosphorylation by LIMK2. On the other hand, it has been reported that LIMK1 is phosphorylated and activated by Pak1 (34). Taken together, these observations suggest that LIMK1 and LIMK2 are regulated by
distinct upstream protein kinases downstream of distinct Rho family GTPases.

LIM-kinase is the LIM domain-containing serine/threonine/tyrosine kinase composed of closely related LIMK1 and LIMK2 (11–17). Recent studies showed that LIMK1 and LIMK2 play a role of actin cytoskeletal reorganization through cofilin phosphorylation downstream of distinct Rho family GTPases (18–20). LIMK1 acts downstream of Rac1 but is not a direct target of this GTPase. It has been shown that LIMK1 is directly phosphorylated and activated by Pak1, an effector protein kinase of Cdc42/Rac and is essential for Pak1-induced dorsal membrane ruffles formation (34, 35). On the other hand, Maekawa et al. (27) showed that ROCK can activate LIMK1 and LIMK2. However, they used the constitutive active form of ROCK (C-terminally deleted ROCK including ROCK-δ3), which resulted in a non-selective activation of LIMK1 and LIMK2. We found that the full-length ROCK specifically activates LIMK2 but not LIMK1. Thus, the C-terminal-half region containing Rho-binding and PH domain has a definite role in the substrate-targeting mechanism for specifying actions of ROCK toward LIMK2. Ohashi et al. (28) reported that wild-type ROCK phosphorylates and activates LIMK1. Although we have no explanation for the discrepancy in all these results, their data contradict the proposal that LIMK1 is specifically regulated by Rac1 but not RhoA and plays a role in the Rac1-induced lamellipodia formation (18–20), as do our present results.

The mechanisms that determine the specificity of Rho-kinase/ROCK action toward its multiple kinase substrates have only been partly characterized. In cells, this kinase, once activated, translocates from the cytosol to the plasma membrane (23). It was also reported that both Rho and Rho-kinase are translocated from the cytosol to the cleavage furrow and play a critical role in inducing and maintaining the contractile ring during cytokinesis (36, 37). The target proteins of Rho-kinase/ROCK, including the ezrin/radixin/moesin family, myosin light chain, myosin binding subunit, glial fibrillary acidic protein, vimentin, and desmin, accumulate at these areas, where their phosphorylation occurs specifically (33, 37–43). However, the dominant active form of Rho-kinase C-terminally deleted form) non-selectively phosphorylates vimentin and desmin in the cytoplasm of interphase cells (42, 43). These observations suggest that the C-terminal region of Rho-kinase containing PH and Rho binding domains may regulate its subcellular localization and, hence, provide susceptibility to specific substrates. The PH domain has a role in the signal-dependent membrane localization of several proteins (44). Rho binding domains of Rho-kinase/ROCK is essential for activation of protein kinase activity through binding to Rho and translocation to the membrane by forming a complex with activated Rho (22, 23). Therefore, even though potential substrates are non-selectively phosphorylated in vitro, susceptibility of Rho-kinase/ROCK to substrates at specific loci may determine if Rho-kinase/ROCK...
ROCK-Δ3

Cofillin

(kDa)

ROCK-Δ3

LIMK2

IgG

Blot : Anti-HA

(kDa)

Blot : Anti-Myc

ROCK-Δ3

LimK2

w.t. T505V T505E

- + - + - +

63 62 47

FIG. 6. Requirement of Thr-505 for activation of LIMK2 by ROCK. COS-7 cells were co-expressed with HA-tagged LIMK2 or its Thr-505 mutants and empty vector or Myc-tagged ROCK-Δ3. After transient expression, immunoprecipitated LIMK2 and its Thr-505 mutants were used for in vitro kinase assay using GST-cofilin as substrate (top panel) and for anti-HA immunoblot (upper middle panel). Cell lysates (50 μg) were also used for anti-Myc immunoblot (lower middle panel). The bottom panel shows changes in protein kinase activity toward cofilin phosphorylation. Protein kinase activity of wild-type (w.t.) LIMK2 in control cells was taken as 1.0. Each value represents the mean ± S.E. of three independent experiments. The arrow indicates autophosphorylated LIMK2.

ROCK-dependent Activation of LIMK2 could act on substrate proteins within cells. Although the intracellular localization of endogenous LIMK2 remains to be determined, all these observations lead to the notion that the activated native form of ROCK may translocate to specific sites where LIMK2 co-localizes, then preferentially phosphorylate LIMK2, the result being LIMK2-dependent cofilin phosphorylation and stabilization of actin filaments.

The spatial and temporal organization of actin filaments plays an essential role in cellular locomotion, and actin cytoskeletal reorganization is distinctly regulated both spatially and temporally within migrating cells (45, 46). In these cells, membrane ruffling and filopodial protrusion, respectively, regulated under Rac and Cdc42 are observed in the leading edge, whereas actin-myosin contraction regulated under Rho is observed in the ruffling area and posterior regions of cells (3–5). Thus, actin cytoskeletal reorganization, which occurs in a distinct spatiotemporal manner in distinct subcellular loci, appears to be regulated through Rho family GTPases, which utilize different sets of downstream effectors. In terms of actin filament depolymerization, independent pathways, i.e. Rac1-Pak1-LIMK1 and RhoA-ROCK-LIMK2, may play a role in distinct regulation of cofilin-mediated actin filament depolymerization such that it occurs in a distinct spatiotemporal manner. Alternatively, different expression patterns of LIMK1 and LIMK2 in tissues and cells (12–17, 47) may specify the tissue- or cell type-specific patterns of actin filament organization.

Acknowledgments—We thank Dr. S. Narumiya (Kyoto University, Kyoto, Japan) for providing plasmids of ROCK and WeFide Co. (Osaka, Japan) for providing Y-27632. We are also grateful to Dr. Y. Takai for helpful discussion and to M. Ohara for helpful comments and for language assistance.

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