LIM-kinase 2 induces formation of stress fibres, focal adhesions and membrane blebs, dependent on its activation by Rho-associated kinase-catalysed phosphorylation at threonine-505

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LIM-kinase 1 and 2 (LIMK1 and LIMK2) phosphorylate cofilin and induce actin cytoskeletal reorganization. LIMK1 is activated by Rho-associated, coiled-coil-forming protein kinase (ROCK) and p21-activated kinase 1 (PAK1), but activation mechanisms and cellular functions of LIMK2 have remained to be determined. We report here that LIMK1 and LIMK2 phosphorylate both cofilin and actin-depolymerizing factor (ADF) specifically at Ser-3 and exhibit partially distinct substrate specificity when tested using site-directed cofilin mutants as substrates. We also show that LIMK2 is activated by ROCK by phosphorylation at Thr-505 within the activation loop. Wild-type LIMK2, but not its mutant (T505V) with replacement of Thr-505 by Val, was activated by ROCK *in vitro* and *in vivo*. LIMK2 mutants with replacement of Thr-505 by one or two Glu residues (T505E or T505EE) increased the kinase activity about 3.6-fold but were not further activated by ROCK. When expressed in HeLa cells, wild-type LIMK2, but not the T505V mutant, induced the formation of stress fibres, focal adhesions and membrane blebs. Furthermore, inhibitors of Rho and ROCK significantly suppressed LIMK2-induced stress fibres and membrane blebs. These results suggest that LIMK2 functions downstream of the Rho-ROCK signalling pathway and plays a role in reorganization of actin filaments and membrane structures, by phosphorylating cofilin/ADF proteins.

Key words: actin-depolymerizing factor, actin reorganization, activation loop, cofilin, LIMK.

INTRODUCTION

The rapid turnover of polymerization and depolymerization of actin filaments underlies actin cytoskeletal reorganization and plays a pivotal role in cell activities, including cell locomotion, morphological change and cytokinesis [1]. Among a variety of actin-binding proteins involved in actin-filament dynamics, cofilin and its closely related protein, actin-depolymerizing factor (ADF, also termed destrin), play an essential role in rapid turnover of actin filaments by depolymerizing and severing actin filaments [1–5]. The activities of ADF/cofilin proteins are reversibly regulated by phosphorylation and dephosphorylation of the serine residue at position 3, with the phosphorylated form being inactive [6,7]. Phosphorylation of ADF/cofilin in living cells is up- or down-regulated by various external cues that stimulate actin reorganization [2,4]. Thus enzymes regulating phosphorylation and dephosphorylation of ADF/cofilin at Ser-3 seem to play important roles in actin-filament dynamics and in actin cytoskeletal remodelling.

LIM-kinases 1 and 2 (LIMK1 and LIMK2) are closely related serine/threonine kinases with structures composed of two N-terminal LIM domains, an internal PDZ domain and a C-terminal protein kinase domain [8–13]. We and others provided evidence that LIMK1 phosphorylates cofilin specifically at Ser-3 *in vitro* and *in vivo* and induces actin cytoskeletal reorganization by phosphorylating and inactivating cofilin [14,15]. LIMK1 is predominantly expressed in developing neural tissues, while LIMK2 is expressed in a variety of developing and adult tissues [8,11]. Distinct patterns of expression suggest that LIMK1 and LIMK2 may play distinct roles in cell activities. Accumulated evidence suggests that the Rho-family small GTPases, Rho, Rac and Cdc42, play a central role in regulating actin reorganization through their various downstream effectors [16–18]. LIMK1 is activated in cultured cells by Rac and Rho, although the activation is indirect [14,15,19]. Recent studies revealed that LIMK1 is activated by Rho-associated, coiled-coil-forming protein kinase (ROCK) and p21-activated kinase 1 (PAK1), which are downstream effectors of Rho and Rac respectively, through phosphorylating Thr-508 within the activation loop in the kinase domain of LIMK1 [19–22]. These findings indicate that both Rac-PAK1 and Rho-ROCK signalling pathways induce activation of LIMK1, which in turn leads to phosphorylation and inactivation of cofilin and reorganization of actin filaments [20,23]. Recent studies suggest that LIMK2 is activated by Rho and ROCK and is involved in stress fibre and focal-adhesion formation [19,24]. However, the molecular mechanism by which LIMK2 is activated downstream of Rho remains to be determined.

We report here that LIMK2 induces formation of stress fibres, focal adhesions and membrane blebs and we also provide evidence that LIMK2 is activated by ROCK *in vitro* and *in vivo*, by phosphorylation at Thr-505 within the activation loop in the kinase domain. This phosphorylation is essential for LIMK2 to induce formation of actin stress fibres and membrane blebs. Our findings suggest that LIMK2, similar to LIMK1, has the potential to regulate actin cytoskeletal reorganization by phosphorylating

Abbreviations used: ADF, actin-depolymerizing factor; HA, haemagglutinin; LIMK, LIM-kinase; PAK, p21-activated protein kinase; PK, protein kinase domain; ROCK, Rho-associated, coiled-coil-forming protein kinase; DMEM, Dulbecco's modified Eagle's medium; DAPI, 4′,6-diamidino-2-phenylindole.

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cofilin/ADF, under the control of the Rho-ROCK signalling pathway. We also characterized the substrate preference of LIMK1 and LIMK2 using site-directed mutants of cofilin.

EXPERIMENTAL

Plasmid construction

Expression plasmid coding for haemagglutinin (HA) epitope-tagged human LIMK1 was constructed in the pUcD2SRα vector, as described in [20]. Plasmids for Myc-tagged ROCKΔ3 (a C-terminally truncated, constitutively active mutant of ROCK, amino acid residues 1–727) and ROCK(KD-IA) (a kinase-defective, Rho-binding-negative mutant of ROCK, with replacements of both Lys-105 and Ile-109 by Ala) were constructed in the pCAG vector, as described in [25]. To construct the expression plasmid coding for N-terminally HA-tagged LIMK2, the full-length human LIMK2 cDNA [11] inserted into pBluescript II(SK$^-$) (Stratagene) at the *Eco*RI site (pBS-LIMK2) was digested with *Sma*I and *Kpn*I, and subcloned into the *Sma*I and *Kpn*I sites of the pUcD2SRα-3HA vector. The cDNA for the kinase-inactive LIMK2(D451A) mutant, in which Asp-451 was replaced by Ala, was constructed, using mutated oligonucleotide 5′-GTTGTGCGAGTTCAGAGCCCGGTGGATGATGCA-3′ and a site-directed mutagenesis kit (Clontech). Plasmid coding for LIMK2(T494A) mutant, in which Thr-494 was replaced by Ala, was constructed by PCR amplification of the cDNA fragment, using primers 5′-TCACAGCAGATCTTCCG-3′ and 5′-TGTAGCGCTTCTTGCGGTCGTTCTTGCGCAAGGC-GCGTTTCTTGGT-3′, and ligation into the *Acc*III and *Eco*47III sites of pUcD2SRα-HA-LIMK2. Plasmids coding for T505A, T505V, T505E and T505EE mutants of LIMK2, in which Thr-505 was replaced by Ala, Val, Glu and two Glu residues respectively, were constructed by PCR amplification of the cDNA fragments, using 5′ primers 5′-AAGAAGCGCTACGCGGT-GGTGGGAAACC-3′, 5′-AAGAAGCGCTACGTGGTGGT-GGGAAACC-3′, 5′-GCAAGAAGCGCTACGAGGTGGTG-GGAAACC-3′ and 5′-GCAAGAAGCGCTACGAAGAAGT-GGTGGGAAACC-3′ respectively, and 3′ primer 5′-ATGC-TCACAGTGTGGTCCAA-3′, and ligation into the *Eco*47III and *Dra*III sites of pUcD2SRα-HA-LIMK2. To construct the plasmid coding for LIMK2-PK [an N-terminally truncated LIMK2 mutant that is mainly composed of the protein kinase domain (PK), amino acid residues 283–639], pBS-LIMK2 was digested with *Axy*I and *Sma*I and subcloned into the *Sma*I site of the pUcD2SRα-3HA vector. Bacterial expression plasmids coding for site-directed mutants of $(His)_6$-tagged cofilin were constructed by PCR amplification of the cDNA fragments, using 5′ primers designed for site-directed sequences and 3′ primer 5′-TTGCTCTTGAGGGGTGCATT-3′, and ligation into the *Nco*I and *Apa*I sites of pQE60-cofilin-$(His)_6$ [7]. The authenticity of expression plasmids was confirmed by nucleotide sequence analysis. Expression plasmid (pEGFP-C1) coding for green fluorescent protein (GFP) was purchased from Clontech.

Cell culture and transfection

COS-7 cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Subconfluent COS-7 cells in a 100 mm dish were transfected with 8 μg of plasmid DNA with DAC30 liposome (Eurogenetec) and cultured for 36–48 h in DMEM supplemented with fetal calf serum. For immunostaining, HeLa cells were plated on 24 mm glass coverslips and cultured for 16 h in DMEM with 10% fetal calf serum, and then cultured for a further 18 h in serum-free DMEM. Cells were transfected with 2 μg of plasmid with Lipofectamine (Gibco-BRL) and cultured for 20 h in serum-free DMEM.

Immunoprecipitation and immunoblotting

COS-7 cells transfected with plasmids coding for HA-tagged LIMK2 or its mutants were washed with ice-cold PBS, suspended in the lysis buffer (50 mM Tris/HCl, pH 7.5/150 mM NaCl/1% Nonidet P-40/10 mM NaF/1 mM Na_3VO_4/1 mM dithiothreitol/1 mM PMSF/10 μg/ml leupeptin), and incubated on ice for 30 min. After centrifugation, supernatants were precleared with Protein A–Sepharose (Amersham Pharmacia Biotech) at 4 °C for 1 h. The supernatants were incubated overnight at 4 °C with 12CA5 anti-HA antibody (Roche Diagnostics) and Protein A–Sepharose. The immunoprecipitates were washed and used for *in vitro* kinase reaction or immunoblot analysis. Immunoblot analysis was carried out as described in [26].

In vitro kinase assay

Immunoprecipitates were washed three times with lysis buffer and then three times with the kinase buffer (20 mM Hepes/NaOH, pH 7.2/5 mM $MgCl_2$/5 mM $MnCl_2$/10 mM NaF/1 mM Na_3VO_4/1 mM PMSF/10 μg/ml leupeptin) and incubated for 30 min at 30 °C in 30 μl of the kinase buffer containing 25 μM ATP, 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) and 2 μg of $(His)_6$-tagged cofilin, ADF or their mutants. $(His)_6$-tagged cofilin, ADF and their mutants were expressed in *Escherichia coli* and purified as described in [15,20]. The reaction mixture was suspended in Laemmli's sample buffer, and aliquots were separated by SDS/PAGE using 15% and 9% gels. Proteins were transferred on to PVDF membranes (Bio-Rad). The membrane from a 15% gel was subjected to autoradiography to measure ^{32}P-labelled cofilin/ADF, using the BAS1800 Bio-Image Analyser (Fuji Film), and Amido Black staining. Membrane from the 9% gel was analysed by autoradiography and immunoblotting with anti-HA antibody to detect LIMK2 or its mutants. The kinase activity was normalized by dividing the radioactivity incorporated into cofilin by the immunoreactive density of LIMK2 estimated by densitometer. For the *in vitro* kinase assay for ROCK-induced activation of LIMK2, immunoprecipitates of HA-tagged LIMK2 or its mutants were mixed with anti-Myc immunoprecipitates from COS-7 cell lysates transfected with pCAG vector or plasmids coding for Myc-tagged ROCKΔ3, and were then incubated for 30 min at 30 °C with 25 μM ATP, 5 μCi of [γ-^{32}P]ATP and 2 μg of $(His)_6$-tagged cofilin and analysed for ^{32}P incorporation into cofilin, as above. ROCKΔ3 was detected by immunoblot analysis with 9E10 anti-Myc antibody. In experiments for ROCK-induced activation of LIMK2 *in vivo*, plasmids coding for HA-tagged LIMK2 or its mutants were co-transfected into COS-7 cells with pCAG vector or plasmids coding for ROCKΔ3. After 36 h, cells were lysed and LIMK2 was immunoprecipitated with anti-HA antibody and subjected to *in vitro* kinase reaction, as described above.

Cell staining

HeLa cells were plated on 24 mm glass coverslips and transfected with plasmid DNA. After 20 h of culture in serum-free DMEM, cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed and permeabilized in 0.2% Triton X-100 in PBS for 5 min. After blocking with 2% fetal calf serum in PBS for 30 min, these cells were incubated with anti-HA polyclonal antibody (provided from Dr Y. Fujiki, Kyushu University, Fukuoka, Japan) for 2 h and subsequently with FITC-conjugated anti-rabbit IgG (Chemicon) for 1 h. Cells were then incubated

with Rhodamine-conjugated phalloidin (Molecular Probes) or 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) for 10 min. To visualize focal adhesions, vinculin was immunostained by anti-vinculin monoclonal antibody (hVIN-1, Sigma), followed by Rhodamine-conjugated anti-mouse IgG (Chemicon). Cells were photographed on a Leica DMLB fluorescence microscope.

RESULTS

Phosphorylation of cofilin and ADF by LIMK1 and LIMK2

Both LIMK1 and LIMK2 were reported to phosphorylate cofilin specifically at Ser-3 [14,15], but their actions on ADF remained to be examined. Based on the structural similarity between cofilin and ADF [2], ADF may possibly serve as substrate for these kinases. As shown in Figure 1, the *in vitro* kinase reaction revealed that both LIMK1 and LIMK2 efficiently phosphorylated wild-type ADF and cofilin, but not their S3A mutants, in which each Ser-3 was replaced by alanine. These findings indicate that both LIMK1 and LIMK2 phosphorylate ADF as well as cofilin, specifically at Ser-3.

Substrate specificity of LIMK1 and LIMK2

We next examined the substrate specificity of LIMK1 and LIMK2 using site-directed mutants of cofilin with replacement of Gly-4, Val-5, Val-7 and Asp-9 by other amino acids. These residues are highly conserved among ADF/cofilin family proteins in various species (Figure 2A) [2]. As shown in Figure 2(B), the *in vitro* kinase reaction revealed that LIMK1 efficiently phosphorylated the G4A, G4L, G4E, G4K and G4F mutants, in which Gly-4 was replaced by Ala, Leu, Glu, Lys and Phe respectively, to the levels of 58–125%, compared with that of wild-type cofilin. In contrast, LIMK2 efficiently phosphorylated the G4A mutant but not G4L, G4E, G4K and G4F. Neither LIMK1 nor LIMK2 phosphorylated the G4P mutant (with replacement of Gly-4 by Pro). Thus substrate containing aliphatic, hydrophobic, acidic and basic amino acids as well as glycine, but not proline, at the +1 position C-terminal to the phosphorylatable Ser-3 can be recognized by LIMK1, whereas LIMK2 seems to have a more stringent substrate specificity, preferring Gly and Ala to other amino acids at the +1 position. LIMK1 efficiently phosphorylated the V5L mutant (with replacement of Val-5 by Leu), but not the V5K and V5F mutants (with replacement of Val-5 by Lys and Phe respectively), whereas LIMK2 efficiently phosphorylated the V5L, V5K and V5F mutants. V5A-cofilin (with replacement of Val-5 by Ala) was only faintly phosphorylated by LIMK1 and LIMK2. Thus, hydrophobic residues (Val or Leu) are favoured at the +2 position of the substrate for both LIMK1 and LIMK2. LIMK1 has more stringent substrate specificity for hydrophobic residues, whereas LIMK2 can recognize substrates containing Lys and Phe as well as Val and Leu at the +2 position. Substitution of Val-7 by Gly (V7G mutant) significantly reduced the phosphorylation by either LIMK1 or LIMK2, indicating that the hydrophobic residue but not the small residue at the +4 position is favoured as the substrate for both LIMKs. Substitution of Asp-9 by Ala (D9A) had no apparent effect on the substrate recognition of LIMKs. Taken together, LIMK1 and LIMK2 commonly prefer the substrate containing hydrophobic residues at the +2 and +4 positions and are unable to recognize the substrate containing Pro at the +1 position. On the other hand, LIMK1 and LIMK2 exhibit a significantly distinct substrate preference; LIMK1 has a stringent preference for hydrophobic residues at the +2 position, as represented by -Ser-(Gly/Ala/Glu/Lys/Phe)-(Val/Leu)-Xaa-(hydrophobic)-, while LIMK2 prefers sequences containing small aliphatic residues at the +1 position, as represented by -Ser-(Gly/Ala)-(Val/Leu/Lys/Phe)-Xaa-(hydrophobic)-.

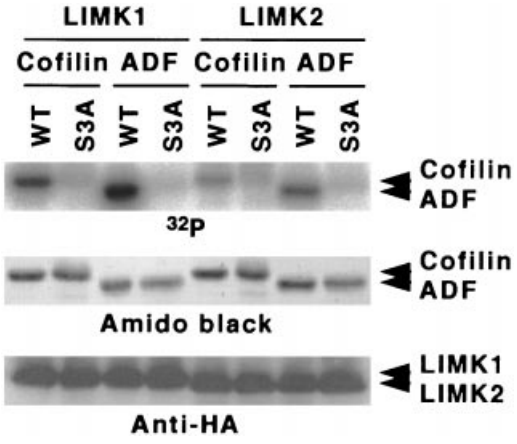

Figure 1 Phosphorylation of cofilin and ADF by LIMK1 and LIMK2

HA-tagged LIMK1 or LIMK2 immunoprecipitated from COS-7 cells were incubated with [γ-^{32}P]ATP and $(His)_6$-tagged wild-type (WT) or the S3A mutant of cofilin or ADF. Reaction mixtures were separated on SDS/PAGE, and subjected to autoradiography (^{32}P), Amido black staining and immunoblotting with anti-HA antibody, as indicated. These results are representative of three independent experiments.

LIMK2 induces formation of actin stress fibres, membrane blebs and focal adhesions

The activity of LIMK2 to phosphorylate ADF/cofilin suggests that it has a role in actin cytoskeletal reorganization. To examine the effect of LIMK2 on actin reorganization, HA-tagged LIMK2 was expressed in HeLa cells and actin filaments were visualized by staining with Rhodamine-labelled phalloidin. Significant changes in actin organization were observed in LIMK2-expressing cells, compared with findings with surrounding LIMK2-non-expressing cells; an increase in actin stress fibres was observed in 64% of the cells expressing LIMK2 (Figure 3A and Table 1), and multiple actin clumps were observed in 37% of the LIMK2-expressing cells (Figure 3B and Table 1). Actin clumps were observed near the apical surface of the LIMK2-expressing cells (Figure 3B). Differential interference contrast (DIC) microscopy revealed further that multiple membrane blebs were formed on the apical surfaces of LIMK2-expressing cells, which resemble membrane morphology that occurs in the execution stage of apoptotic cells [27]. A higher magnification revealed that staining of actin filaments was concentrated at the base of blebs, not within the blebs, whereas LIMK2 immunofluorescence was concentrated uniformly in the blebs (Figure 3B , lower panels). In contrast, expression of a kinase-inactive mutant of LIMK2, LIMK2(D451A), in which the catalytic residue Asp-451 was replaced by Ala, had no apparent effect on the actin cytoskeleton and membrane morphology (Figure 3A, lower panels). Stress fibres and membrane bleb formation were also seen in cells expressing LIMK1, but LIMK1 was less effective in inducing stress fibres and membrane blebs (Table 1), and rather it induced distinct patterns of polymerized actin structures at the cell periphery, as we reported elsewhere [15].

We also examined effects of LIMK2 expression on focal-adhesion formation. Immunostaining for vinculin, a major com-

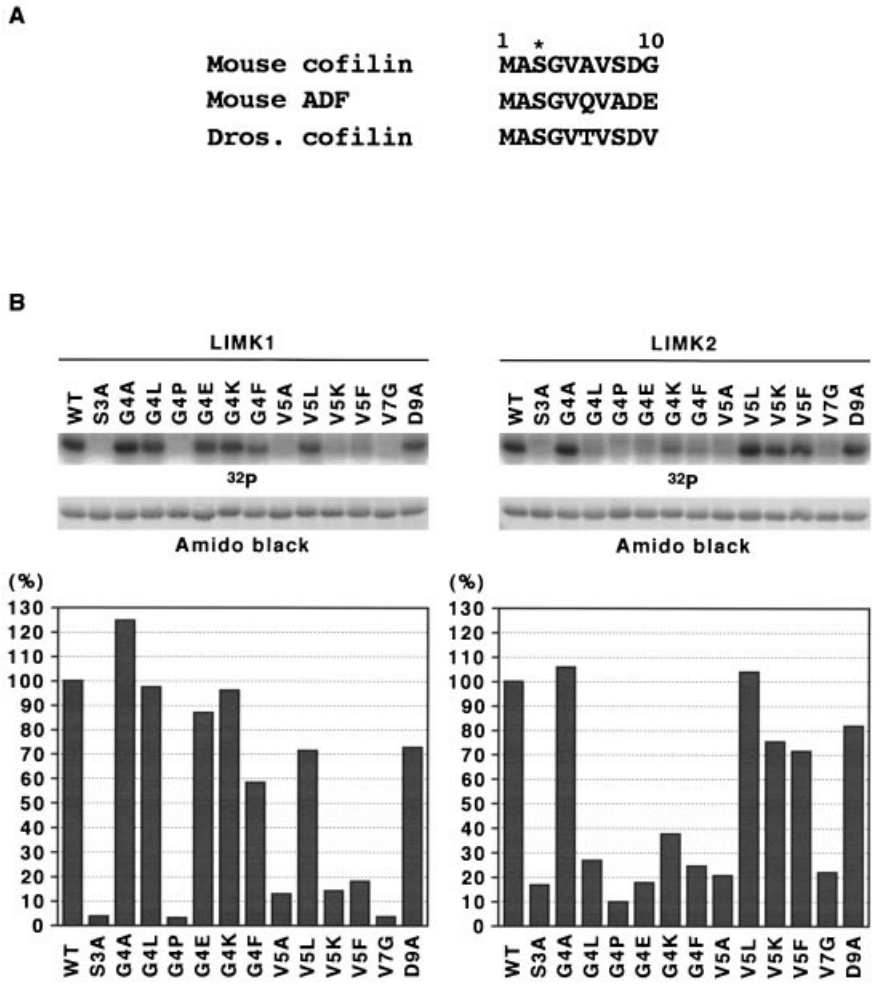

Figure 2 Substrate specificity of LIMK1 and LIMK2

(**A**) Alignment of N-terminal sequences of mouse cofilin, mouse ADF and *Drosophila* (Dros.) cofilin. The asterisk indicates the position of phosphorylatable Ser-3. (**B**) Substrate specificity of LIMK1 and LIMK2. $(His)_6$-tagged mouse cofilin (WT) and its site-directed mutants were incubated with [γ-^{32}P]ATP and HA-tagged LIMK1 or LIMK2, and analysed as in Figure 1. Lower panels indicate the relative values of ^{32}P-incorporation into cofilin mutants, taking the value of wild-type cofilin as 100%. Results are representative of two independent experiments.

ponent of focal adhesions, revealed that LIMK2 strengthened the formation of focal adhesions at cell margins (Figure 3C, upper two panels). We also observed enhanced vinculin immunofluorescence in membrane blebs at the apical surfaces of LIMK2-expressing cells. The pattern of vinculin distribution is similar to patterns of focal-adhesion proteins, focal-adhesion kinase (FAK) and paxillin, which are concentrated in apoptotic blebs [28]. Expression of a kinase-negative LIMK2(D451A) did not enhance focal-adhesion formation (Figure 3C, lower two panels). Thus LIMK2 induces the formation of actin stress fibres, membrane blebs and focal adhesions in a manner dependent on its kinase catalytic activity. LIMK2 overexpression induced 'apoptosis-like' membrane blebbing, but not chromatin condensation/fragmentation, one of the hallmarks of apoptosis, as measured by staining with DAPI (results not shown).

Co-expression of S3A-cofilin or S3A-ADF (a non-phosphorylatable and constitutively active form of cofilin or ADF) with LIMK2 suppressed LIMK2-induced formation of actin stress fibres, membrane blebs (Figure 3D) and focal adhesions (results not shown), suggesting that LIMK2 induces actin stress fibres, focal adhesions and membrane blebs through phosphorylation and inactivation of cofilin/ADF proteins.

ROCK activates LIMK2 by phosphorylation at Thr-505

Our earlier studies revealed that ROCK activates LIMK1 through phosphorylation at Thr-508 [20], but the mechanism of LIMK2 activation by ROCK remained to be determined. As reported [19], kinase activity of LIMK2 was 16.7-fold increased by incubation with an active form of ROCK, ROCKΔ3, as measured by cofilin-phosphorylating activity (Figure 4A). Incubation with a kinase-negative form of ROCK, ROCK(KD-IA), had no apparent effect on the kinase activity of LIMK2 (results not shown), suggesting that LIMK2 is activated by ROCK as a result of phosphorylation. Indeed, the level of phosphorylation of LIMK2 increased after incubation with ROCKΔ3 (Figure 4A). To determine mechanisms of ROCK-catalysed LIMK2 activation, we constructed several mutants of LIMK2 and measured their kinase activities *in vitro* in the

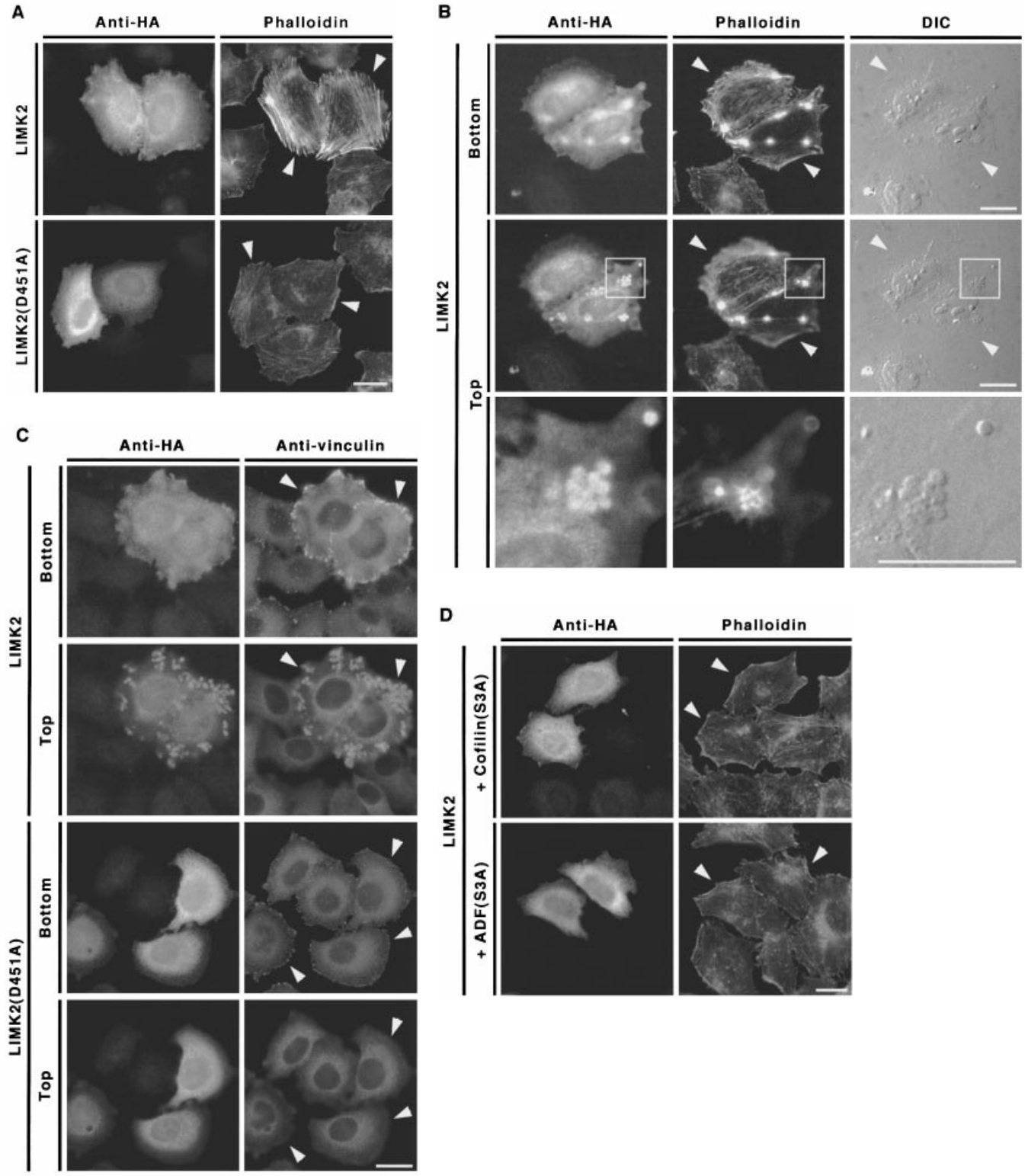

Figure 3 LIMK2 induces the formation of actin stress fibres (A), membrane blebs (B) and focal adhesions (C), and S3A-cofilin and S3A-ADF suppress the actions of LIMK2 (D)

(**A**–**C**) HeLa cells were transfected with plasmids coding for HA-tagged LIMK2 or its kinase-inactive D451A mutant. Cells were stained with anti-HA antibody, Rhodamine-conjugated phalloidin and anti-vinculin antibody, as indicated. Differential interference contrast (DIC) images of the cells are also shown in (**B**). The fluorescence and differential interference contrast images focused near the bottom and top levels of the cells are shown in (**B**) and (**C**). Higher-magnification images of the top levels of the cells are also shown in the third row in (**B**). The areas magnified are shown by the white squares in the middle row. Arrowheads indicate cells expressing LIMK2 or LIMK2(D451A). (**D**) HeLa cells were co-transfected with plasmids coding for HA-tagged LIMK2 and plasmids for S3A-cofilin or S3A-ADF. Cells were stained with anti-HA antibody and Rhodamine-conjugated phalloidin, as indicated. These results are representative of three independent experiments. Scale bars, 10 μm.

Table 1 Effects of expression of LIMK1, LIMK2 or LIMK2 mutants on formation of stress fibres and membrane blebs

HeLa cells were transfected with expression plasmids coding for the indicated protein and stained with anti-HA antibody and Rhodamine-conjugated phalloidin. The number of the cells in which staining of stress fibres had increased compared with that in surrounding non-transfected cells, and in which membrane blebs had been induced, was counted and the percentage of these cells in the HA-positive cells was calculated. Green fluorescent protein (GFP) was used as a control. The results of triplicate experiments are shown as means ± S.E.M.

	Cells with stress fibres induced (% of transfected cells)	Cells with membrane blebs induced (% of transfected cells)
GFP	2.8 ± 0.21	0.6 ± 0.31
LIMK1 (wild type)	38.2 ± 5.2	5.2 ± 0.2
LIMK2 (wild type)	64.1 ± 1.89	36.8 ± 3.62
LIMK2(D451A)	2.2 ± 1.65	0.4 ± 0.33
LIMK2(T505V)	5.4 ± 1.12	0.3 ± 0.25
LIMK2(T505E)	63.9 ± 5.5	4.4 ± 0.49
LIMK2(T505EE)	70.1 ± 1.97	7.6 ± 0.26
LIMK2-PK	67.4 ± 2.18	40.9 ± 1.02

absence or presence of ROCKΔ3. The C-terminal PK of LIMK2 had 2.8-fold higher kinase activity than the wild-type LIMK2 in the absence of ROCKΔ3 (Figure 4A). By incubation with ROCKΔ3, PK was phosphorylated and the kinase activity of PK increased further by about 17.6-fold, which is comparable with the 16.7-fold increase in the kinase activity of full-length LIMK2 by ROCKΔ3. Thus ROCKΔ3 seems to activate LIMK2 by phosphorylating the C-terminal PK domain. The kinase-inactive D451A mutants of LIMK2 and the PK fragment, used as controls, were phosphorylated by ROCKΔ3 but had no kinase activity, even after treatment with ROCKΔ3 (Figure 4A). We also observed phosphorylation of LIMK2 and PK, but not LIMK2(D451A) and PK(D451A), by incubation in the absence of ROCKΔ3, which suggests that LIMK2 and PK have the potential to undergo autophosphorylation.

Many protein kinases are activated by phosphorylation of the residue(s) within the activation loop in the kinase domain [29]. To determine the site of phosphorylation responsible for activation of LIMK2, mutations were introduced at two threonine residues (Thr-494 and Thr-505), which are conserved within the activation loop among human, rat, mouse and chicken LIMK2 [9,11–13]. As shown in Figure 4(B), LIMK2(T494A), with replacement of Thr-494 by Ala, was phosphorylated and activated by ROCKΔ3, to an extent similar to the phosphorylation and activation of wild-type LIMK2 treated with ROCKΔ3. In contrast, LIMK2(T505A), with replacement of Thr-505 by Ala, was neither phosphorylated nor activated by ROCKΔ3. These results clearly demonstrate that ROCKΔ3 activates LIMK2 by phosphorylating Thr-505 within the activation loop of LIMK2.

To further assess the role of Thr-505 phosphorylation for the kinase activity of LIMK2, we constructed LIMK2 mutants with replacement of Thr-505 by Val (T505V), a non-phosphorylatable

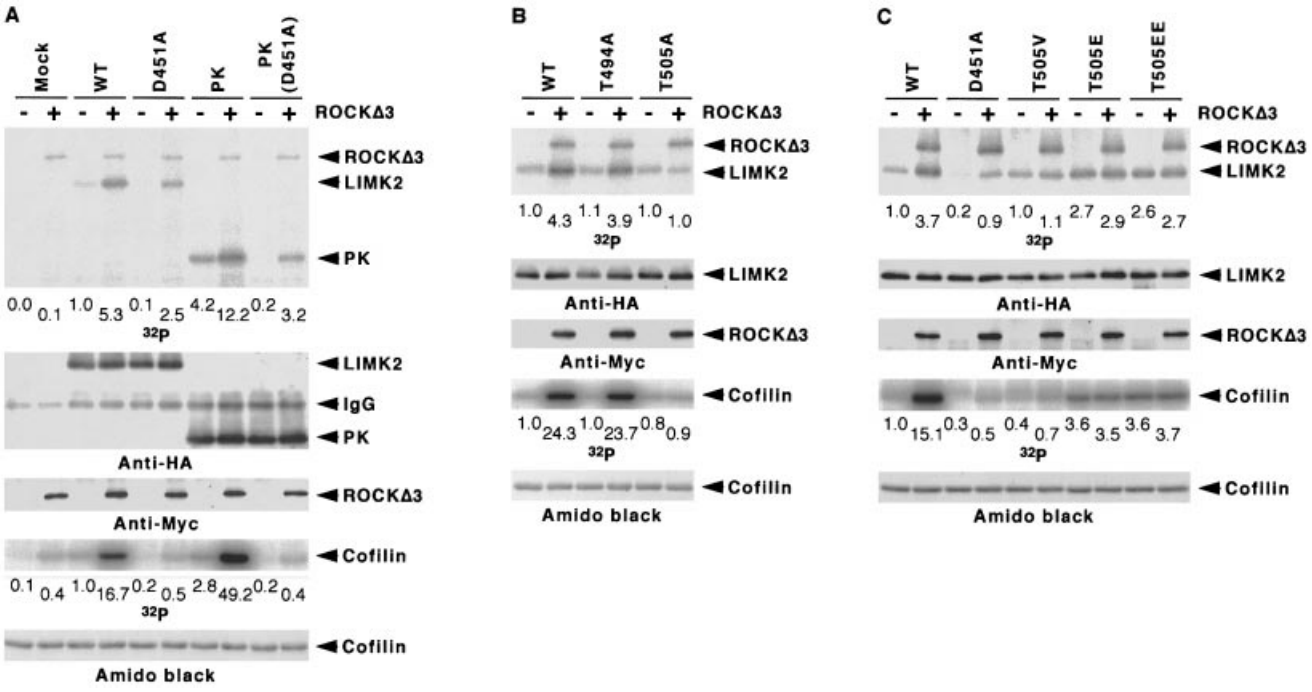

Figure 4 LIMK2 is activated by ROCK through phosphorylation of Thr-505

HA-tagged wild-type (WT) LIMK2 and its various mutants were expressed in COS-7 cells, immunoprecipitated with anti-HA antibody, mixed with anti-Myc immunoprecipitates from COS-7 cell lysates transfected with pCAG empty vector (−) or plasmid coding for Myc-tagged ROCKΔ3 (+), and then subjected to *in vitro* kinase reaction, using $(His)_6$-tagged cofilin as a substrate. Reaction mixtures were separated on SDS/PAGE (9 and 15% gels) and transferred on to PVDF membranes. The membrane from the 9% gel was subjected to autoradiography to detect phosphorylation of LIMKs (top panel) and immunoblotting with anti-HA (second panel down) and anti-Myc antibody (third panel down) to detect LIMKs and ROCKΔ3, respectively. The membrane from the 15% gel was autoradiographed to detect phosphorylation of cofilin (fourth panel down) and Amido black staining for cofilin (bottom panel). The kinase activity, as measured by ^{32}P-incorporation into cofilin (fourth panel), was normalized by the density of immunoreactivity of HA-tagged LIMKs (second panel). The relative kinase activities of LIMK2 mutants are indicated under the fourth panel, taking the activity of LIMK2 without ROCKΔ3 as 1.0. The relative levels of ^{32}P-incorporation into LIMK2 mutants are indicated under the top panel, also taking the level of wild-type LIMK2 without ROCKΔ3 as 1.0. Experiments were repeated twice, and similar results were obtained.

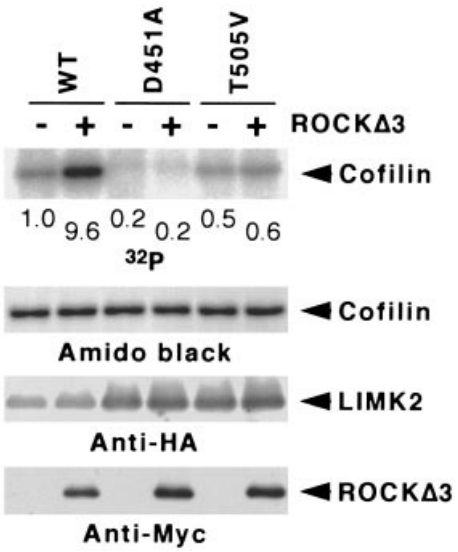

Figure 5 ***In vivo*** **activation of LIMK2 by ROCK**

COS-7 cells were transfected with pUcD2 plasmids coding for HA-tagged wild-type LIMK2 (WT) or its D451A or T505V mutants and either pCAG vector (−) or that for Myc-tagged ROCKΔ3 (+). HA-tagged LIMK2 and its mutants were immunoprecipitated with anti-HA antibody, incubated with [γ-^{32}P]ATP and $(His)_6$-tagged cofilin, and run on SDS/PAGE on 15 and 9% gels. The membrane from the 15% gel was subjected to autoradiography (top panel) and Amido black staining (second panel down), and the membrane from the 9% gel was immunoblotted with anti-HA antibody (third panel down). Relative kinase activities, as measured by ^{32}P-incorporation into cofilin, are indicated under the top panel. Expression of ROCKΔ3 was also analysed by immunoprecipitation and immunoblotting with anti-Myc antibody (bottom panel). Experiments were repeated twice, and similar results were obtained.

residue with a size comparable with that of threonine, or by one or two glutamic acids (T505E or T505EE), which were expected to mimic the phosphorylated state of threonine. The kinase activity of the T505V mutant was reduced compared with that of wild-type LIMK2, and was not enhanced by incubation with ROCKΔ3 (Figure 4C). The T505E and T505EE mutants had an approx. 3.6-fold higher kinase activity than wild-type LIMK2, but incubation with ROCKΔ3 did not increase the kinase activity further (Figure 4C). These results confirm further that LIMK2 is activated by ROCKΔ3 through Thr-505 phosphorylation.

Activation of LIMK2 by ROCK in cultured cells

We next asked if ROCK could activate LIMK2 in cultured cells by phosphorylating Thr-505. LIMK2 and its D451A and T505V mutants were expressed in COS-7 cells, with or without ROCKΔ3, and their kinase activities were measured after immunoprecipitation. The kinase activity of wild-type LIMK2 was about 10-fold increased by co-expression with ROCKΔ3, but no significant increase in the kinase activity of the T505V and D451A mutants was observed (Figure 5). These results suggest that ROCKΔ3 activates LIMK2 *in vivo* as well as *in vitro* by phosphorylation of Thr-505.

Phosphorylation of Thr-505 is required for LIMK2-induced stress fibre and membrane bleb formation

We next examined effects of expression of Thr-505 mutants of LIMK2 on actin reorganization in HeLa cells, in order to determine if Thr-505 phosphorylation is required for the activity of LIMK2 to induce stress fibres and membrane blebs (Figure 6 and Table 1). As described above, expression of wild-type LIMK2

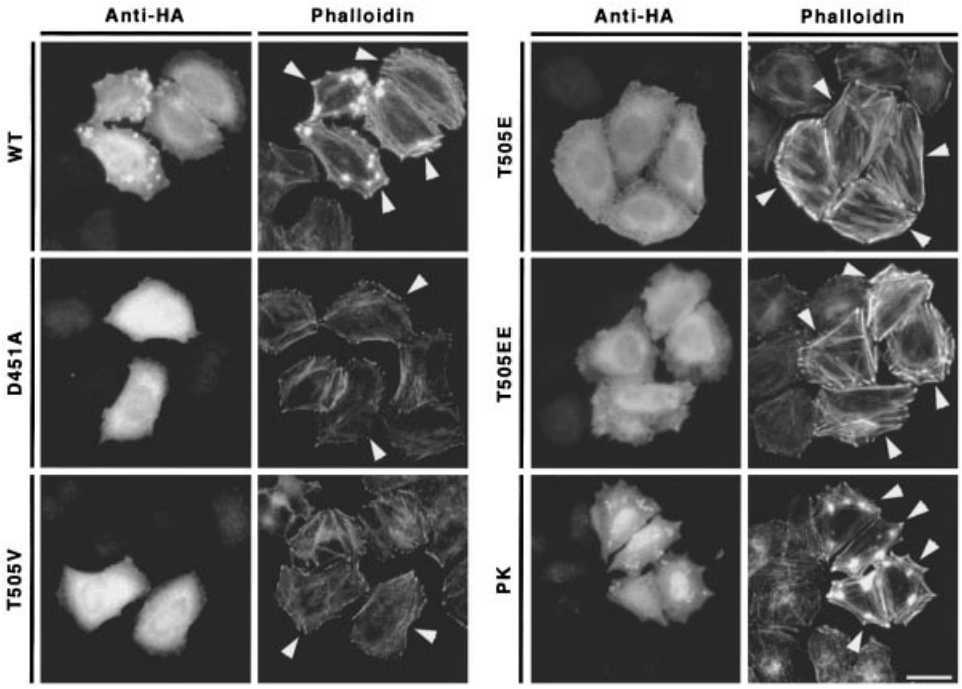

Figure 6 Phosphorylation of Thr-505 is required for LIMK2-induced actin reorganization

HeLa cells were transfected with plasmids coding for HA-tagged wild-type (WT) LIMK2 or its mutants, as indicated. Cells were stained with anti-HA antibody or Rhodamine-conjugated phalloidin. Arrowheads indicate cells expressing LIMK2 or its mutants. These results are representative of three independent experiments. Scale bar, 10 μm.

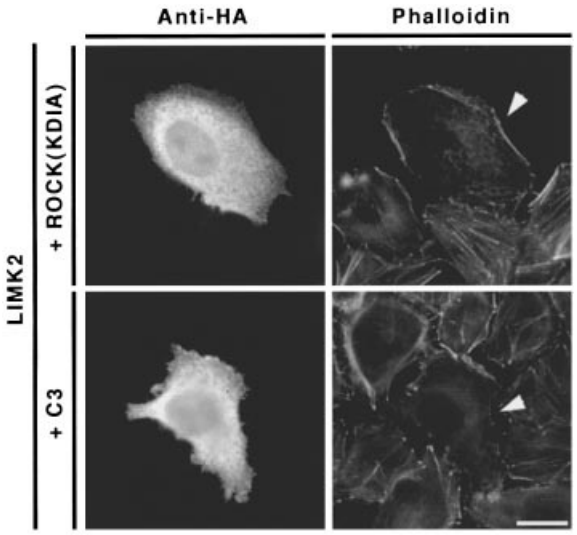

Figure 7 Effects of ROCK(KD-IA) and C3 on LIMK2-induced actin reorganization

HeLa cells were co-transfected with plasmids for HA-tagged LIMK2 and Myc-tagged ROCK(KD-IA) or C3. Cells were stained with anti-HA antibody or Rhodamine-conjugated phalloidin. Experiments were repeated twice, and similar results were obtained. Scale bar, 10 μm.

induced the formation of stress fibres and membrane blebs in 64% and 37% of the transfected cells, respectively. Similar extents of stress fibre and membrane bleb formation were observed in the cells expressing the PK domain of LIMK2, indicating that the PK domain is sufficient for LIMK2-induced changes in actin and membrane organization. On the other hand, the T505V mutant failed to induce the formation of stress fibres and membrane blebs, which is comparable with the lack of effect of a kinase-inactive D451A mutant or control green fluorescent protein. Thus phosphorylation of Thr-505 seems to be essential for the activity of LIMK2 to induce stress fibre and membrane bleb formation. Expression of LIMK2(T505E) and LIMK2(T505EE) mutants significantly induced the formation of stress fibres, and these fibres were thicker than those induced by wild-type LIMK2. On the other hand, membrane blebs were induced in only a small population (4–8%) of the cells expressing these mutants (Table 1). These results suggest that T505E and T505EE mutants cannot completely mimic an active Thr-505-phosphorylated LIMK2, which probably has additional functions other than cofilin phosphorylation to induce membrane blebs.

Rho/ROCK signalling is required for LIMK2-induced stress-fibre and membrane-bleb formation

We next examined the effects of inhibitors of Rho and ROCK on the LIMK2-induced stress-fibre and membrane-bleb formation. As shown in Figure 7, co-expression of a dominant-negative mutant of ROCK, ROCK(KD-IA), resulted in a significant loss of LIMK2-induced actin stress fibres and membrane blebs. Similar results were obtained when LIMK2 was co-expressed with *Clostridium botulinum* C3 toxin, an enzyme that specifically inactivates Rho by ADP-ribosylation [30]. Immunostaining with anti-vinculin antibody revealed that co-expression with ROCK(KD-IA) or C3 toxin abolished the LIMK2-induced focal-adhesion formation (results not shown). These results suggest that the Rho-ROCK signalling pathway is required for the LIMK2-induced stress-fibre, membrane-bleb and focal-adhesion formation.

We further examined the effect of Y-27632, a specific inhibitor of ROCK [31], on LIMK2-induced actin rearrangement, in order to determine the short-term effects of ROCK inhibition. Stress fibres and membrane blebs induced in LIMK2-expressing cells disappeared with a 60 min treatment of the cells with 10 μM Y-27632 (Figure 8A, upper panels). Immunostaining with anti-vinculin antibody revealed that focal adhesions in LIMK2-expressing cells were also reduced by treatment with Y-27632 (results not shown). Treatment of the LIMK1- or LIMK2-expressing cells with 10 μM Y-27632 for 30–120 min significantly reduced the kinase activity of LIMK1 or LIMK2 in cells, whereas the kinase activity of T505V or T505E mutant of LIMK2 in transfected cells was not changed after treatment of the cells with Y-27632 (Figures 8B and 8C). On the other hand, in *in vitro* assays Y-27632 at concentrations of 10–200 μM did not affect the kinase activity of LIMK1 and LIMK2 but inhibited the activity of ROCKΔ3 to stimulate LIMK1/LIMK2 activity (results for LIMK2 are shown in Figure 8D). These observations suggest further that LIMK activity in cells depends on the kinase activity of ROCK. Interestingly, when LIMK2(T505E)-transfected cells were treated with Y-27632, stress fibres and membrane blebs disappeared, but lamellipodium-like polymerized actin structures were observed at cell peripheries (Figure 8A, lower panels). These results suggest that a constitutively active LIMK2(T505E) by itself has the potential to induce polymerized actin structures, and that LIMK2 activation without active ROCK induces distinct types of polymerized actin structures other than stress fibres.

DISCUSSION

ADF/cofilin proteins play a critical role in regulating actin-filament dynamics by promoting depolymerization and severance of actin filaments [1–5]. As the activities of ADF/cofilin are abolished by phosphorylation at Ser-3, protein kinases responsible for this phosphorylation probably play important roles in regulating actin-filament turnover and thereby actin cytoskeletal reorganization. We and others reported that LIMK1 and LIMK2 phosphorylate cofilin *in vivo* and *in vitro* and induce actin reorganization [14,15]. In the present study, we show that both LIMK1 and LIMK2 phosphorylate ADF as well as cofilin. LIMK1- and LIMK2-induced actin reorganization is abolished by co-expression with a constitutively active form of cofilin or ADF (S3A-cofilin or S3A-ADF). These findings suggest that LIMK1 and LIMK2 induce actin reorganization by phosphorylating and inactivating cofilin/ADF proteins. Under our experimental conditions, cofilin and ADF behaved similarly in the cell activity and sensitivity to LIMK1 and LIMK2. Thus these proteins seem to be isoforms with similar activities, although subtle differences between them in *in vitro* behaviour, regulation of expression and subcellular localization have been reported [4].

Expression of LIMK2 induced the formation of actin stress fibres, membrane blebs and focal adhesions in HeLa cells. The morphology of polymerized actin and plasma membrane structures was slightly different from the morphology induced by LIMK1; actin filaments accumulated in the cell periphery in most of the LIMK1-expressing cells [14,15], and membrane blebs were detected only in 5% of LIMK1-expressing cells, in contrast to 35% of LIMK2-expressing cells (Table 1). Thus LIMK2 seems to play a role, overlapped with but partially distinct from that of LIMK1, in actin reorganization and membrane dynamics.

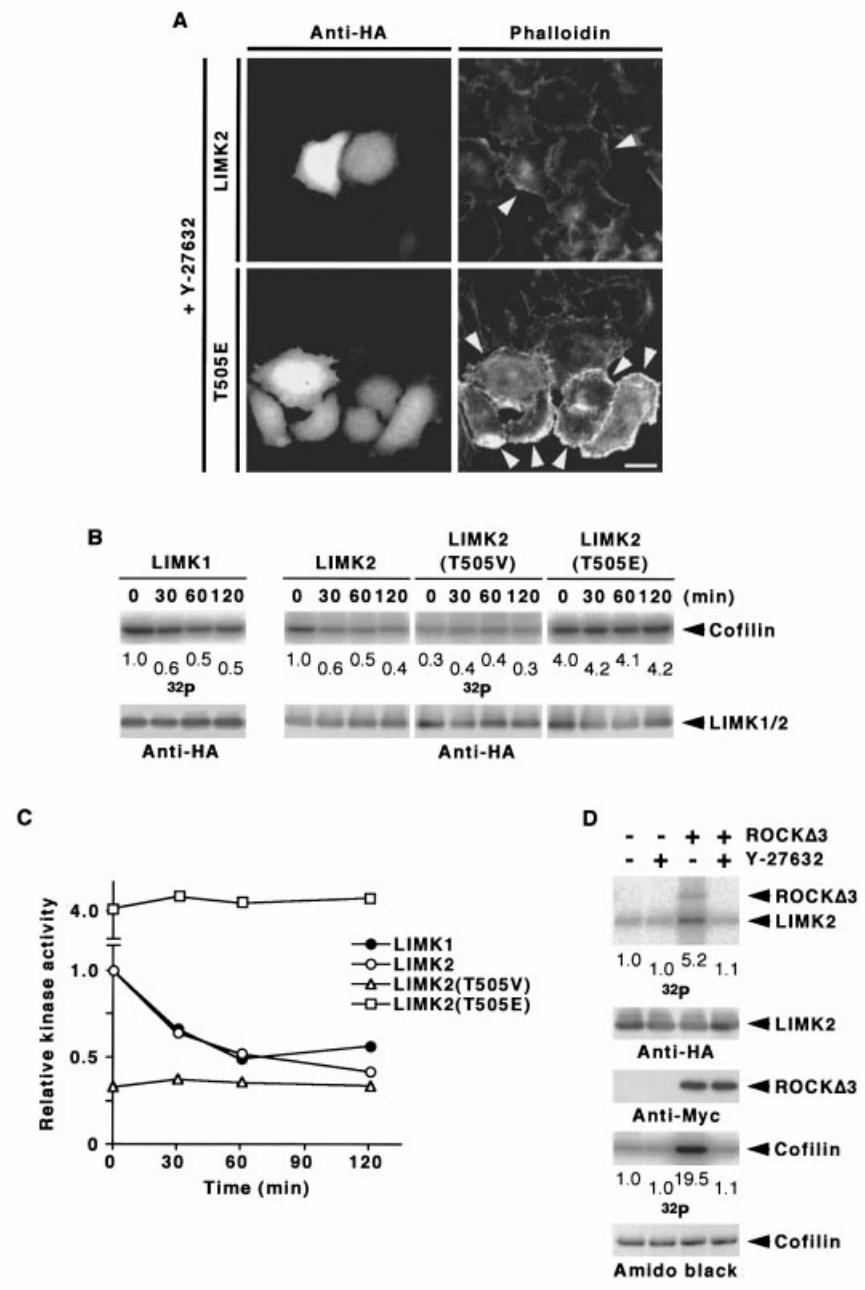

Figure 8 Effects of Y-27632 on LIMK2-induced actin reorganization and on *in vitro* and *in vivo* kinase activity of LIMK2

(**A**) HeLa cells were transfected with plasmids coding for HA-tagged wild-type LIMK2 or its T505E mutant. After 1 day of culture, cells were treated with 10 μM Y-27632 for 60 min, then stained with anti-HA antibody and Rhodamine-conjugated phalloidin. Arrowheads indicate cells expressing LIMK2 or its T505E mutant. Scale bar, 10 μm. (**B**) HeLa cells transfected with HA-tagged LIMK1, LIMK2 or LIMK2 mutants were treated with 10 μM Y-27632 for 0–120 min, and then cell lysates were immunoprecipitated with anti-HA antibody and subjected to *in vitro* kinase reaction, using $(His)_6$-tagged cofilin as a substrate. (**C**) Time courses of changes in kinase activity of LIMK1, LIMK2 and LIMK2 mutants after treatment of cells with Y-27632, as measured by ^{32}P-incorporation into cofilin. Relative kinase activities are plotted, taken the activity of LIMK1 or LIMK2 at 0 min as 1.0. (**D**) Effects of Y-27632 on the kinase activity of LIMK2 *in vitro*. HA-tagged LIMK2 was immunoprecipitated, preincubated with 200 μM Y-27632 for 10 min in the presence or absence of ROCKΔ3 and subjected to *in vitro* kinase reaction. Reaction mixtures were analysed as in Figure 4. These experiments were repeated twice, and similar results were obtained.

Since LIMK1 and LIMK2 commonly phosphorylate cofilin/ADF, the distinct patterns of actin filament and membrane organization induced by these kinases may be due to differences in their substrate(s) other than cofilin/ADF or their regulators. We compared the substrate specificities of LIMK1 and LIMK2 using site-directed mutants of cofilin. Both kinases prefer substrates containing hydrophobic residues at positions +2 and +4 C-terminal to the phosphorylatable serine and failed to recognize the substrate containing proline at +1, but they exhibit distinct substrate preference at the +1 and +2 positions (Figure 2). Thus LIMK1 and LIMK2 may have their own as-yet-uncharacterized substrate(s). Alternatively, LIMK1 and LIMK2 may interact with distinct proteins that differentially regulate the activity or subcellular localization of these kinases.

Similar to LIMK1 [21,26], the kinase activity of LIMK2 was enhanced by deleting the N-terminal region containing LIM and PDZ domains, indicating that the N-terminal region plays an inhibitory role in the regulation of the kinase activity of LIMK2. As proposed for many protein kinases [32], activation may occur by interaction of activator proteins with the extracatalytic region to set the kinase domain free; the binding partners for LIM and PDZ domains of LIMKs remain to be found. The PK fragment was activated further by ROCK-catalysed phosphorylation, to a ratio comparable with the activation of full-length LIMK2, which suggests that the kinase activity of LIMK2 is regulated by two independent mechanisms, release from negative regulation by the N-terminal extra-catalytic region and phosphorylation of the C-terminal kinase domain.

Using various mutants of LIMK2, we have shown that LIMK2 is activated by ROCK through phosphorylation of Thr-505 within the activation loop in the kinase domain. We and others reported that LIMK1 is activated by ROCK and PAK1 through phosphorylation of Thr-508 of LIMK1 [19–22]. Residues surrounding Thr-505 of LIMK2 and Thr-508 of LIMK1 (DRKKRYTVVGNP) in the activation loop are completely conserved in LIMK1 and LIMK2 sequences and match consensus sequence motifs for the phosphorylation sites by ROCK, $(R/K)X_{0-2}(S/T)$, and PAK1, (K/R)RX(S/T) [33,34]. Thus it is most probable that LIMK1 and LIMK2 are commonly activated by ROCK and PAK1 by phosphorylation of the conserved Thr-508 and Thr-505 in the activation loop, respectively. It will be of interst to determine if multiple members of the ROCK and PAK family protein kinases and their related kinases, including myotonic dystrophy protein kinase (DMPK), myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), citron kinase and germinal centre kinase (GCK) [35,36], also function as LIMK-activating kinases.

Actin stress fibres were induced by wild-type LIMK2 and its T505E and T505EE mutants, but not by the T505V mutant, which indicates that activation of LIMK2 by Thr-505 phosphorylation is critical for its function to induce actin reorganization. In addition, our findings that LIMK2-induced stress fibres and membrane blebs were repressed by co-expression of C3 exoenzyme or ROCK(KD-IA) or by treatment of the cells with Y-27632 suggest that the Rho-ROCK signalling pathway is essential for functions of LIMK2 to evoke stress fibres and membrane blebs. On the other hand, Y-27632 treatment of the cells expressing a constitutively active LIMK2(T505E) led to formation of lamellipodium-like actin organization at the cell periphery, rather than stress-fibre formation. This would suggest that an active form of LIMK2 has the potential to induce actin reorganization, even under conditions in which ROCK is inhibited by Y-23632, and that activation of LIMK2 alone will not induce stress fibres. It is probable that actions of ROCK other than LIMK2 activation, such as the increase in myosin light-chain phosphorylation through myosin phosphatase inactivation [37], are important for induction of stress fibres. Previous studies suggest that Rho and Rac mutually antagonize cellular activity and that the balance between Rho and Rac activity in cells determines the patterns of actin organization [38,39]. Thus lamellipodium-like actin organization in LIMK2(T505E)-expressing cells treated with Y-27632 may occur due to a preference of basal Rac activity against Rho activity, as a result of inhibition of ROCK by Y-27632 treatment, in which LIMK2(T505E) is constitutively active and independent on the Rho-ROCK pathway.

By co-expression analysis, Sumi et al. [24] recently reported that LIMK1 was activated by Rac but not by Rho and Cdc42, whereas LIMK2 was activated by Rho and Cdc42 but not Rac. They also showed that Rac-induced lamellipodium formation was inhibited by the kinase-dead form of LIMK1, but not the kinase-dead form of LIMK2, whereas Rho-induced stress-fibre formation was inhibited by the kinase-dead form of LIMK2, but not the kinase-dead form of LIMK1 [24]. Based on these observations they suggested that LIMK2 functions downstream of Rho, but not Rac, while LIMK1 functions downstream of Rac, but not Rho [24]. In this study we showed evidence for the molecular basis for the activation of LIMK2 by the Rho-ROCK signalling pathway by identifying Thr-505 as the site of phosphorylation responsible for activation. We earlier reported that LIMK1 is also activated by ROCK *in vitro* and *in vivo* through phosphorylation of the conserved threonine (Thr-508) within the activation loop [19,20]. In addition, lysophosphatidic acid, an activator of the Rho-ROCK signalling pathway, induced activation of LIMK1 and this activation was inhibited by Y-27632 [19,20]. Furthermore, kinase activities of LIMK1 and LIMK2 expressed in HeLa cells were inhibited by treatment of the cells with Y-27632, indicating that they are activated in cells by endogenous ROCK (Figure 8). Based on our results we speculate that LIMK1 and LIMK2 play overlapping roles in actin reorganization, at least under control of the Rho-ROCK pathway, and distinct phenotypes of actin organization induced by Rac and Rho are probably due to differences in downstream effectors of Rac and Rho other than LIMKs. The reason for the discrepancy is not known at present. Further studies will be required to assess the functional difference between LIMK1 and LIMK2 and its physiological importance.

Membrane blebbing occurs in cells undergoing mitosis and apoptosis. The pattern of F-actin staining with phalloidin differs between mitotic and apoptotic blebs; the blebs during mitosis are stained in a uniform manner, whereas apoptotic blebs are stained only at their base, the bleb itself being devoid of F-actin staining [27]. The blebs observed in LIMK2-expressing cells are likely to be those observed in cells during apoptosis. Using C3 exoenzyme and inhibitors of myosin light-chain kinase, Mills et al. [40] showed that activation of Rho and myosin light-chain phosphorylation are critical for membrane blebbing occurring during the execution phase of apoptosis. In this study we showed that LIMK2 induces membrane blebbing that is blocked by C3 exoenzyme and a dominant-negative form of ROCK. Thus activation of LIMK2 downstream of the Rho-ROCK pathway is also involved in apoptotic membrane-bleb formation. Caspase inhibitors block the chromatin condensation and nucleosome laddering but not membrane blebbing, which suggests that membrane blebbing, one of the execution steps of apoptosis, is regulated independently of the caspase-induced pathways [40,41]. In accord with this, LIMK2 induces membrane blebbing but not other execution steps such as chromatin condensation and nucleosome laddering. These results suggest that apoptotic membrane blebbing is co-ordinately regulated by Rho and its multiple downstream effectors. LIMK2 may support the actomyosin contractile structures at the bases of blebs by stabilizing actin filaments by phosphorylating and inactivating the actin-depolymerizing activities of cofilin/ADF proteins.

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