

# The N-terminal LIM domain negatively regulates the kinase activity of LIM-kinase 1

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LIM-kinase 1 (LIMK1, where LIM is an acronym of the three gene products Lin-11, Isl-1 and Mec-3) is a serine/threonine kinase that phosphorylates cofilin and regulates actin cytoskeletal reorganization. LIMK1 contains two LIM domains and a PDZ (an acronym of the three proteins PSD-95, Dlg and ZO-1) domain in the N-terminal half and a kinase domain in the C-terminal half. In this study we examined the role of the extra-catalytic region in the regulation of kinase activity of LIMK1. Limited proteolysis of LIMK1 resulted in the production of the 35–40-kDa kinase core fragments with 3.5–5.5-fold increased kinase activity. The LIMK1 mutants with deleted LIM domains ( $\Delta$ LIM) or conserved cysteines in the two LIM domains replaced with glycines (dmLIMK1) had 3–7-fold higher kinase activities *in vitro*, compared with the wild-type LIMK1. The C-terminal

kinase fragment of LIMK1 bound to the LIM domain but not to the PDZ domain. Furthermore, the LIM fragment dose-dependently inhibited the kinase catalytic activity of the kinase core fragment of LIMK1. Taken together, these results suggest that the N-terminal LIM domain negatively regulates the kinase activity of LIMK1 by direct interaction with the C-terminal kinase domain. In addition, expression of the  $\Delta$ LIM mutant in cultured cells induced punctate accumulation of actin filaments, an event distinct from the pattern of actin organization induced by expression of the wild-type LIMK1, suggesting that the LIM domain plays a role in the function of LIMK1 *in vivo*.

**Key words:** actin cytoskeleton, LIM domain, LIM-kinase, protein kinase, PDZ domain.

## INTRODUCTION

Control of actin-filament dynamics is important in various cell activities, including motility, adhesion, cell-shape determination and cytokinesis [1]. Cofilin, a potent regulator of actin-filament dynamics, can bind to and depolymerize actin filaments [2,3]. Its actin-binding and -depolymerizing activity is abolished by phosphorylation at Ser-3 [4]. We and another group recently provided evidence that LIM-kinase 1 (LIMK1, where LIM is an acronym of the three gene products Lin-11, Isl-1 and Mec-3) phosphorylates cofilin specifically at Ser-3 and is involved in the regulation of actin-filament dynamics by phosphorylating and inactivating cofilin [5,6].

LIMK1 and its related kinase, LIM-kinase 2 (LIMK2), constitute a novel subclass of serine/threonine kinases, possessing characteristic structural features composed of two tandemly arranged LIM domains (LIM1 and LIM2) and a PDZ domain (where PDZ is an acronym of the three proteins PSD-95, Dlg and ZO-1) in the N-terminal half and a kinase catalytic domain in the C-terminal half [7–11]. The LIM domain is composed of paired zinc fingers with a conserved placement of cysteine and histidine residues ( $CX_2CX_{17-19}HX_2C$ ) $X_2(CX_2CX_{16-20}CX_{2-3}C/H/D)$  [12–14]. The LIM domain is found singly or multiply in a diverse set of proteins, including homeodomain-containing transcription factors, cytoskeletal-component proteins and other signalling molecules, which are known to be involved in cell-fate determination, growth regulation, oncogenesis and cytoskeletal reorganization [12–14]. The PDZ domain is a 80–100-amino acid motif found in various sub-membranous and cytoplasmic

proteins, and several PDZ-containing proteins are known to function in the clustering of ion channels and receptors by binding of the PDZ domain to the C-terminal S/TXV tripeptide motifs [15]. As the LIM and PDZ domains are considered to function in protein–protein interactions [12–15], these domains in LIMKs are likely to be involved in regulating kinase activity or subcellular localization through intra- or intermolecular protein interactions. The LIM domain of LIMK1 was found to associate with several isoforms of protein kinase C and the cytoplasmic regions of transmembrane neuregulin isoforms [16,17]. However, the physiological importance of these interactions remains to be determined. The PDZ domain of LIMK1 was found to contain two functional leucine-rich nuclear-export signals, which support the preferentially cytoplasmic localization of LIMK1 [18,19], whereas the binding partner for the PDZ domain of LIMK1 is not yet known.

LIMK1 is expressed predominantly in the nervous systems of developing and adult mammals, suggesting a role for this kinase in neuronal development and functions [7–11,20]. Genetic analyses suggested that hemizygotic deletion of the LIMK1 gene causes impaired visuospatial constructive cognition in patients of Williams syndrome [21]. Expression of LIMK1 was also detected in various cultured cells, including fibroblasts, and epithelial and haematopoietic cell lines [7]. As LIMK1 phosphorylates cofilin and induces actin reorganization, it probably has a role in regulating diverse cell activities, including cell motility, cytokinesis and neurite extension/retraction, through the phosphorylation of cofilin.

Enzyme activity of most protein kinases is strictly controlled

Abbreviations used: GST, glutathione S-transferase; HA, haemagglutinin; LIM, an acronym of the three gene products Lin-11, Isl-1 and Mec-3; LHX, LIM-homeodomain protein; LIMK, LIM-kinase; LMO, LIM-only protein; PDZ, an acronym of the three proteins PSD-95, Dlg and ZO-1; LDB1/NLI, LIM-domain-binding protein/nuclear LIM interactor.

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within the cell. These kinases are usually kept in an inactive state in the absence of activation stimuli and become activated in response to activation signals [22–24]. In many cases, the extra-catalytic region within the kinase molecule serves to repress the kinase activity, and activation stimuli release this repression through events such as activator binding or phosphorylation [22–24]. We reported that the N-terminal region of LIMK1 containing two LIM domains can interact with the remaining C-terminal region [25]. However our report did not provide evidence for the role of this interaction in the regulation of the kinase catalytic activity of LIMK1; we did not know of a practical substrate such as cofilin for measuring the kinase activity of LIMK1. We now have evidence that the LIM domain negatively regulates the kinase activity of LIMK1 by direct interaction with the C-terminal kinase catalytic region of the enzyme. We also noted the activity in actin-cytoskeletal reorganization of a LIMK1 mutant without the LIM domain.

## EXPERIMENTAL

### Plasmid construction

Expression plasmids coding for human LIMK1 and its mutants ( $\Delta$ LIM and PK) and their C-terminally haemagglutinin (HA)-tagged counterparts were constructed in the pUCD<sub>2</sub>SR $\alpha$  vector, as described in [5,18]. The plasmid coding for dmLIMK1, with replacements of Cys-49 and Cys-108 with glycines, was constructed by point mutations of LIMK1 cDNA, using an *in vitro* site-directed mutagenesis kit (Clontech). The plasmid coding for GST–PDZ, glutathione S-transferase (GST) fused with the PDZ domain of LIMK1, was constructed in the pGEX-4T-3 vector, as described previously [18]. The plasmid coding for GST–LIM was constructed by ligation of PCR-amplified cDNA fragments coding for GST and the LIM region of LIMK1 (amino acid residues 22–140) and subcloning into the pET-17b vector (Novagen).

### Antibodies

Rabbit polyclonal antibody, C-10, was raised against the C-terminal 10-amino acid peptide (GLPAHPEVPD) of LIMK1, as described in [10]. Antibody K-19 was raised against a 19-amino acid peptide (RLMVDEKTQPEGLRSLKRP, residues 483–501) within the kinase domain of LIMK1 in a similar manner.

### Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with 14  $\mu$ g of plasmid DNA/100-mm dish by the calcium phosphate method.

### Expression and preparation of LIMK1 and its mutants

COS-7 cells were transfected with plasmids for LIMK1 or its mutants and then incubated for 36–48 h. These cells were washed with ice-cold PBS, suspended in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 5% glycerol, 1 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 10 mM NaF, 1 mM NaVO<sub>4</sub>) containing 1 mM PMSF, 1 mM benzamide and 10  $\mu$ g/ml leupeptin, and incubated on ice for 1 h. After centrifugation, lysates were precleared with Protein A–Sepharose (15  $\mu$ l of 50% slurry; Amersham Pharmacia Biotech) for 1 h at 4 °C. The precleared supernatants were incubated overnight at 4 °C with anti-LIMK1 antibody (C-10) and Protein A–Sepharose (15  $\mu$ l of 50% slurry). After centrifugation, the immunoprecipitates were washed three times with the lysis buffer con-

taining protease inhibitors, and then LIMK1 or its mutants were eluted with the lysis buffer containing C-10 peptide (30  $\mu$ g/ml), placed on ice and used for kinase reaction *in vitro* and immunoblot analysis.

### Kinase assay *in vitro*

LIMK1 or its mutants, eluted with the lysis buffer containing C-10 peptide, were subjected to *in vitro* kinase reaction by incubation with the lysis buffer containing 50  $\mu$ M ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) and (His)<sup>6</sup>-cofilin (0.25 mg/ml) at 30 °C for 45 min [5]. The reaction mixture was suspended in Laemmli's sample buffer and aliquots were separated by SDS/PAGE (15 or 9% gels). Proteins were transferred on to PVDF membranes (Bio-Rad). The membrane from a 15% gel was analysed by autoradiography to measure <sup>32</sup>P-labelled cofilin using a BAS1500 BioImage Analyser and Image Gauge V3.01 software (with Fuji film). The membrane from a 9% gel was analysed by immunoblotting with C-10 or K-19 anti-LIMK1 antibodies. The kinase activity was usually normalized by dividing the radioactivity incorporated into cofilin by the immunoreactive density of LIMK1 estimated by densitometer.

### Immunoblot analysis

For immunoblot analysis, the PVDF membrane was blocked overnight with 5% non-fat dried milk in PBS containing 0.05% Tween 20, and incubated for 2 h at room temperature with C-10 or K-19 anti-LIMK1 antibodies diluted in PBS containing 1% non-fat dried milk and 0.05% Tween 20. After washing in PBS containing 0.05% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech). Immunoreactive protein bands were visualized using an ECL chemiluminescence reagent (Amersham Pharmacia Biotech).

### Limited proteolysis

LIMK1 was prepared from lysates of LIMK1-transfected COS-7 cells by immunoprecipitation with C-10 anti-LIMK1 antibody, as described above. For trypsin digestion, LIMK1 was eluted by the lysis buffer containing C-10 peptide (30  $\mu$ g/ml), and digested by the addition of 25  $\mu$ g/ml trypsin at 30 °C. The reaction was stopped by adding 1 mg/ml soya bean trypsin inhibitor, then the mixture was subjected to kinase reaction *in vitro*. For lysyl endopeptidase digestion, LIMK1 immunoprecipitates were washed twice with the lysis buffer and digested with 25  $\mu$ g/ml lysyl endopeptidase (Wako Pure Chemicals) in the same buffer at 30 °C. The reaction was stopped by washing three times with ice-cold lysis buffer containing 2 mM *p*-tosyl-L-lysine chloromethyl ketone, 1 mM benzamide and 10  $\mu$ g/ml leupeptin. LIMK1 fragments were eluted from Sepharose beads by the lysis buffer containing C-10 peptide and then subjected to kinase reaction *in vitro*.

### Expression and purification of GST-fusion proteins

The plasmids coding for GST-fusion proteins were transformed into *Escherichia coli* XL1-Blue. An overnight bacterial culture in selective medium was diluted 1:10 into fresh medium. After a 1-h pre-culture at 37 °C, isopropyl  $\beta$ -D-thiogalactopyranoside was added to the culture to a final concentration of 0.5 mM, and then incubated for another 3 h at 37 °C for induction of GST and GST–PDZ. For the induction of GST–LIM protein, the bacteria were cultured at 18 °C for 5 h, collected and suspended in the lysis buffer containing protease inhibitors. The suspensions were

sonicated and cleared by centrifugation at 12000 *g* for 10 min. The supernatants were added to glutathione–Sepharose (15  $\mu$ l of 50% slurry; Amersham Pharmacia Biotech), and incubated overnight at 4 °C. The beads were washed three times with the lysis buffer and used for protein-binding analysis. To measure the kinase inhibitory activity, GST and GST–LIM were eluted with 10 mM glutathione in the lysis buffer and added into the kinase reaction mixture.

### Cell staining

Cells were plated on 24-mm glass coverslips and transfected with the plasmids for HA-tagged LIMK1 or  $\Delta$ LIM. After 36 h, cells were fixed and permeabilized, as described in [18, 19]. To visualize the expression of HA-tagged proteins, cells were stained with anti-HA polyclonal antibody (Babco) followed by FITC-labelled anti-rabbit IgG antibody (ICN Pharmaceuticals). Cells were also stained for F-actin with 0.2 unit/ml Rhodamine-conjugated phalloidin (Wako Pure Chemicals) for 10 min at room temperature. Fluorescence was viewed and photographed using an epifluorescent microscope (Carl Zeiss).

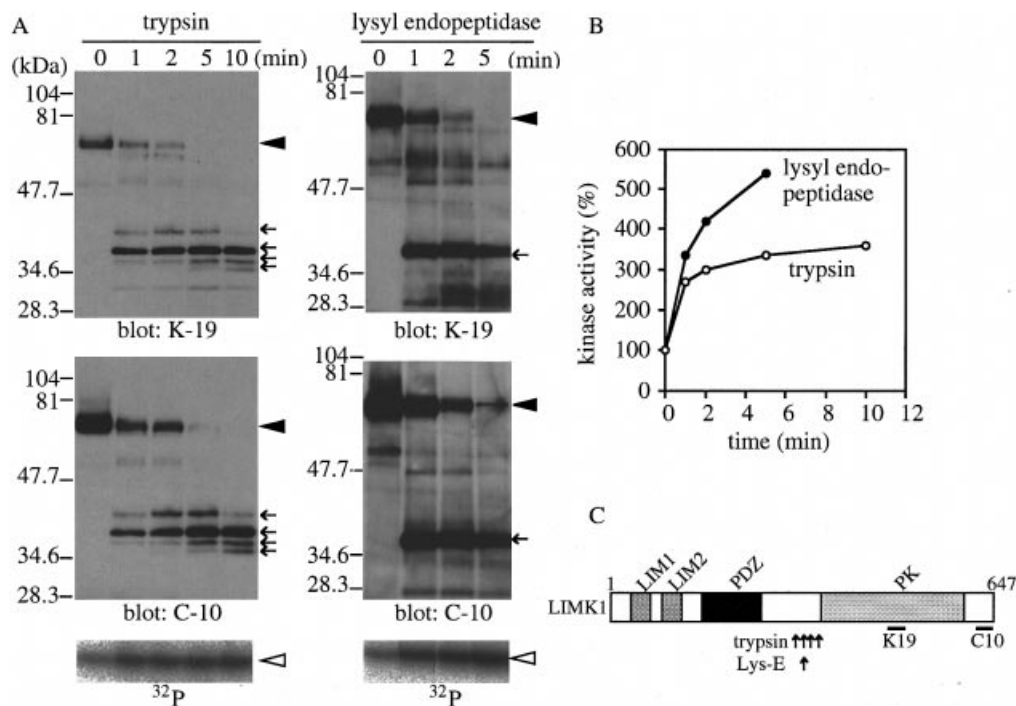
## RESULTS

### Effects of limited proteolysis on the kinase activity of LIMK1

To understand the role of the extra-catalytic region of LIMK1 in kinase activity, LIMK1 was prepared by immunoprecipitation and subjected to limited proteolysis. The reaction products were

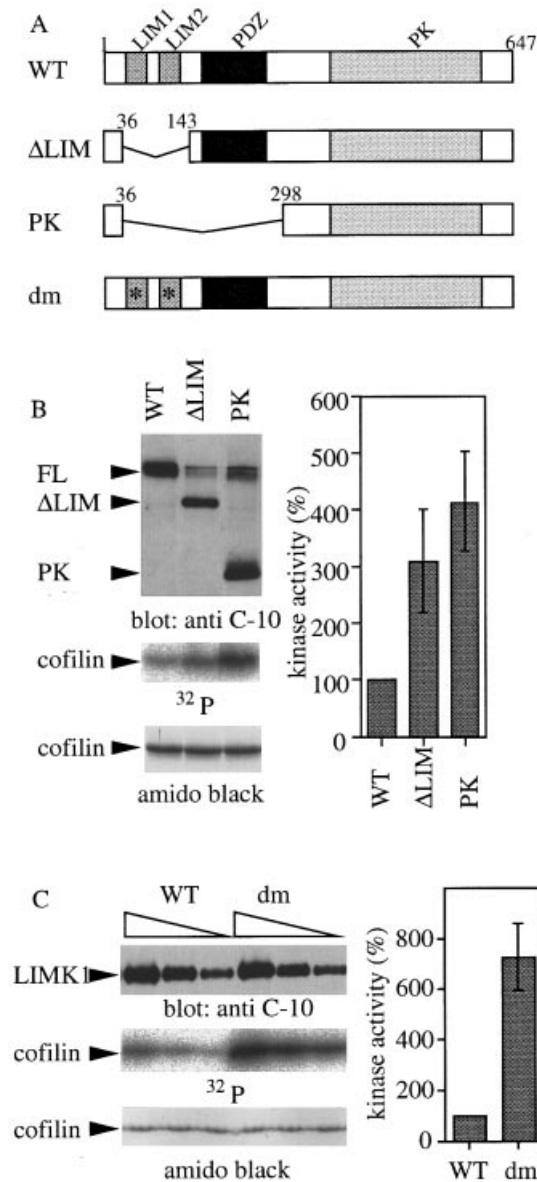
then analysed for activity of this kinase, using recombinant cofilin as a substrate. Treatment of LIMK1 with trypsin decreased the amount of full-length intact LIMK1 and inversely increased the production of proteolytic fragments, with apparent molecular masses of 40, 37, 36 and 35 kDa (Figure 1A, left panel). These fragments were recognized by the antibody raised against the C-terminal peptide (C-10) as well as the antibody raised against the peptide within the kinase domain (K-19). Based on the molecular sizes and immunoreactivities towards both C-10 and K-19 antibodies, it was estimated that the 35–40-kDa fragments were the C-terminal fragment derived by cleavage at sites present in the serine-rich joint region (residues 295–332) between the PDZ domain and the kinase domain (Figure 1C). Trypsin digestion resulted in a significant increase in the cofilin-phosphorylating activity of LIMK1 (maximally 3.5-fold). The time-dependent increase in the kinase activity of LIMK1, shown in Figure 1(B), correlated well with the appearance of LIMK1 C-terminal fragments of 35–40 kDa.

To further define the site of cleavage, LIMK1 was treated with lysyl endopeptidase, which has strict substrate specificity towards the C-terminal side of lysyl residues. Similar to the case of trypsin digestion, treatment of LIMK1 with lysyl endopeptidase resulted in the time-dependent production of a 37-kDa fragment of LIMK1 with immunoreactivity towards both C-10 and K-19 antibodies (Figure 1A, right panel). Based on size of the fragment and specificity of the protease, the cleavage site was estimated to be Lys-316. The time-dependent increase (maximally 5.5-fold) in the kinase activity correlated with production of a 37-kDa



**Figure 1** Increase in the kinase activity of LIMK1 by limited proteolysis

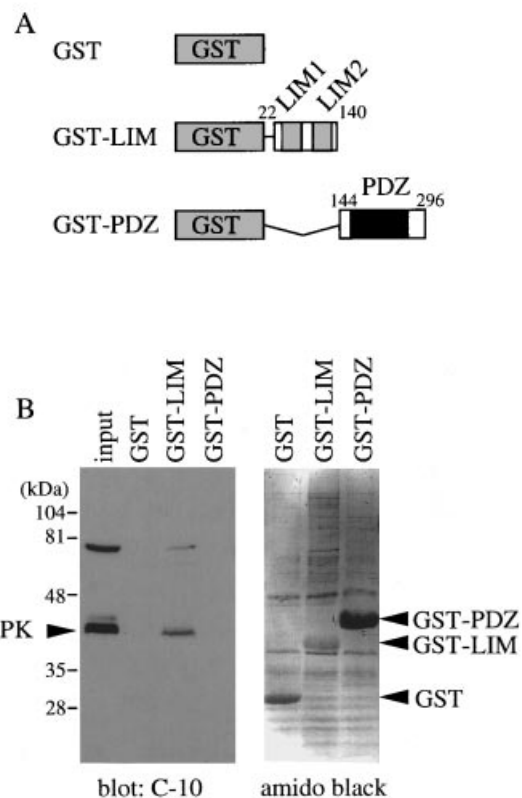
(A) LIMK1 was digested with trypsin or lysyl endopeptidase, as described in the Experimental section. At the indicated times, the reaction was stopped, and the reaction mixtures were subjected to kinase reaction *in vitro* and separated by SDS/PAGE (9 and 15% gels). The 9% gel was analysed for protein cleavage by Western blots, using K-19 and C-10 anti-LIMK1 antibodies (upper and middle panels, respectively), whereas the 15% gel was analysed for kinase activity by measuring <sup>32</sup>P incorporation into cofilin (lower panels). Closed arrowheads indicate the positions of intact 70-kDa LIMK1. Arrows indicate the positions of the 35–40-kDa proteolytic fragments of LIMK1. Open arrowheads indicate the positions of cofilin. (B) Time courses of changes in the kinase activity of LIMK1 during limited proteolysis. The kinase activity, as measured by <sup>32</sup>P incorporation into cofilin, was plotted by taking the activity of non-treated LIMK1 (at 0 min) as 100%. (C) Schematic representation of the structure of LIMK1, composed of two LIM domains (LIM1 and LIM2), a PDZ domain (PDZ) and a protein kinase domain (PK). Predicted cleavage sites with trypsin and lysyl endopeptidase (Lys-E) are indicated by arrows. Recognition sites for K-19 and C-10 anti-LIMK1 antibodies are indicated by underlines. Residue numbers are indicated at the top of the diagram.



**Figure 2** Deletion or mutation of LIM domains activates LIMK1

(A) Diagrams showing structures of wild-type (WT) LIMK1 and its mutants. The numbers above the deleted mutants indicate amino acid residues flanking the deleted regions of LIMK1. Asterisks in dm indicate the sites of point mutations, where Cys-49 and Cys-108 were replaced by glycines. (B) Kinase activity of LIMK1 and its deleted mutants. LIMK1 immunoprecipitates were eluted by C-10 peptide and subjected to kinase reaction *in vitro*. The kinase activity, measured by  $^{32}\text{P}$  incorporation into cofilin (middle panel), was normalized by the density of immunoreactivity with C-10 antibody (upper panel). Lower panel indicates amido black staining of cofilin. The activity of wild-type LIMK1 was taken as 100%. FL, full-length LIMK1. (C) Kinase activity of various doses of wild-type and dmLIMK1. The kinase activity was measured and calculated as in (B). Data in the right panels (B and C) are the means  $\pm$  S.D. of triplicate experiments.

fragment (Figure 1B). These results suggest that the C-terminal-half region of LIMK1 containing the protein-kinase domain resists proteolytic digestion. The observation that loss of the N-terminal half by limited proteolysis significantly increased the kinase activity of LIMK1 suggests that the N-terminal region negatively regulates the kinase activity of LIMK1.



**Figure 3** Interaction between the N-terminal LIM and the C-terminal PK fragment of LIMK1

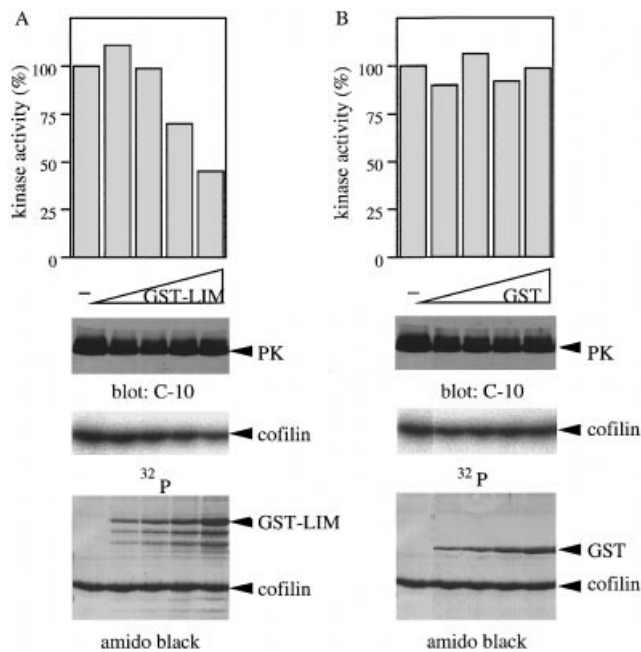
(A) Diagrams showing structures of GST-fusion proteins. The numbers above the diagrams indicate amino acid residues of human LIMK1. (B) Co-precipitation binding assay of PK with GST-fusion protein. PK prepared from lysates of COS cells transfected with the PK plasmid was incubated with GST, GST-LIM, or GST-PDZ bound to glutathione-Sepharose beads. After washing, the bound proteins were solubilized and subjected to SDS/PAGE, followed by immunoblot analysis with C-10 antibody (left panel) and by amido black staining (right panel). Arrowheads indicate the positions of PK and GST-fusion proteins. The 'input' lane shows the immunoblot of PK used in the binding assay.

#### Kinase activity of N-terminally deleted mutants of LIMK1

To examine further the role of the N-terminal region in the kinase activity of LIMK1, we constructed expression plasmids coding for a LIMK1 mutant lacking the LIM domains ( $\Delta$ LIM) or the one lacking both LIM and PDZ domains (PK; Figure 2A). LIMK1 and its deleted mutants were expressed in COS cells and tested for their cofilin-phosphorylating activities following immunoprecipitation with C-10 antibody. As shown in Figure 2(B),  $\Delta$ LIM and PK mutants exhibited about 3–4-fold higher kinase activities compared with the full-length wild-type LIMK1. These results suggest further that the N-terminal region, particularly the LIM domain, has the potential to suppress the kinase activity of LIMK1.

#### Kinase activity of a LIMK1 mutant with point mutations within the LIM domains

To define further the role of the LIM domain, we expressed a mutant (dmLIMK1) of full-length LIMK1 with double point mutations replacing two cysteines (Cys-49 and Cys-108) in the



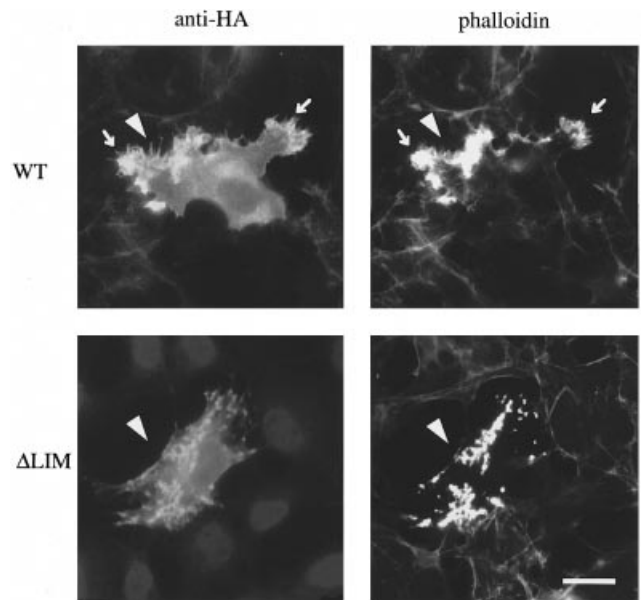
**Figure 4** Kinase activity of PK is inhibited by the LIM domain

PK mutant expressed in COS cells was immunoprecipitated with C-10 antibody and eluted with C-10 peptide. PK was preincubated for 30 min on ice, without (—) or with increasing amounts (32, 63, 125 and 250  $\mu$ g/ml) of GST-LIM (A) or control GST (B), then subjected to kinase reaction *in vitro*. The kinase activity of PK was measured and calculated as in Figure 2(B), by taking the activity in the absence of GST or GST-LIM as 100%. Immunoblots of PK with C-10 antibody, <sup>32</sup>P incorporation into cofilin, and amido black staining of cofilin, GST and GST-LIM are shown in lower panels.

LIM1 and LIM2 motifs with glycines (Figure 2A). These mutations were expected to destroy the zinc-co-ordinated tertiary structure of LIM domains [26]. When the kinase activity of dmLIMK1 was compared with that of the wild-type LIMK1, the former exhibited about a 7-fold higher kinase activity (Figure 2C). Activation of LIMK1 by mutations of conserved cysteine residues in the LIM domains suggests that the zinc-co-ordinated tertiary structure is required for the inhibitory function of the LIM domain with regard to regulating the kinase activity of LIMK1.

#### The LIM domain binds directly to the C-terminal protein-kinase domain

We reported earlier that an HA-tagged LIMK1 mutant ( $\Delta$ LIM-HA) lacking the LIM domain (with residues 34–144 deleted) bound to GST-LIM( $\Delta$ 145–610), a GST-fusion protein of LIMK1 with residues 145–610 deleted (see Figures 1 and 4 in [25]). To exclude the possibility that the N-terminal short sequence (residues 1–21) or the C-terminal short sequence (611–617) in GST-LIM( $\Delta$ 145–610) is involved in the interaction with  $\Delta$ LIM-HA, we constructed GST-LIM(22–140), a GST-fusion protein containing only the LIM region (residues 22–140) of LIMK1 (Figure 3A). When GST-LIM(22–140) was immobilized on glutathione-Sepharose and incubated with the PK fragment of LIMK1 (PK, see Figure 2A), PK bound to GST-LIM(22–140) (Figure 3B). In contrast, PK did not bind to control GST or GST-PDZ containing the PDZ domain of LIMK1. Thus the N-terminal LIM domain interacts directly with the C-terminal kinase domain.



**Figure 5** Expression of LIMK1 and  $\Delta$ LIM in COS cells induces distinct patterns of actin reorganization

COS-7 cells were transfected with plasmids for HA-tagged LIMK1 or  $\Delta$ LIM, and stained with anti-HA antibody (left) and Rhodamine-conjugated phalloidin (right). Arrowheads indicate the cells that express LIMK1 or  $\Delta$ LIM, as assigned by anti-HA immunostaining. Arrows indicate the protruding edges of LIMK1-expressing COS cells. Scale bar, 20  $\mu$ m. WT, wild type.

To examine the requirement of the tertiary structure of the LIM domain for this interaction, we attempted to obtain GST-dmLIM, a GST-fusion protein of the LIM domain of LIMK1 with point mutations of Cys-49 and Cys-108 to glycines. However, this attempt was not productive, since this mutant was unstable in *E. coli* and only degraded products were obtained.

#### The LIM domain inhibits the kinase activity of the PK fragment

We next asked if GST-LIM would inhibit kinase activity of the C-terminal kinase fragment of LIMK1. As shown in Figure 4, the cofilin-phosphorylating activity of PK was reduced significantly when we added increasing amounts of GST-LIM. At the highest dose, GST-LIM inhibited the kinase activity of the PK fragment to below 50%. In contrast, the addition of control GST had no apparent effect, even at the highest dose. These findings further support the proposal that the LIM domain inhibits the kinase catalytic activity of the C-terminal region of LIMK1, by direct interaction.

#### Actin reorganization induced by the LIMK1 mutant without the LIM domain

We and others have reported that expression of LIMK1 in cultured cells induced marked accumulation of actin filaments [5,6]. In a similar manner, when LIMK1 plasmids were transfected into COS-7 cells and actin filaments were visualized by Rhodamine-phalloidin staining, a marked increase in actin filaments was observed in LIMK1-expressing cells, as compared with surrounding LIMK1-non-expressing cells (Figure 5, upper panels). Actin filaments accumulated at the cell periphery and protruding ends of LIMK1-expressing cells (arrows in Figure 5). When  $\Delta$ LIM, a LIMK1 mutant with the LIM domain deleted, was expressed in COS-7 cells, remarkable changes in actin

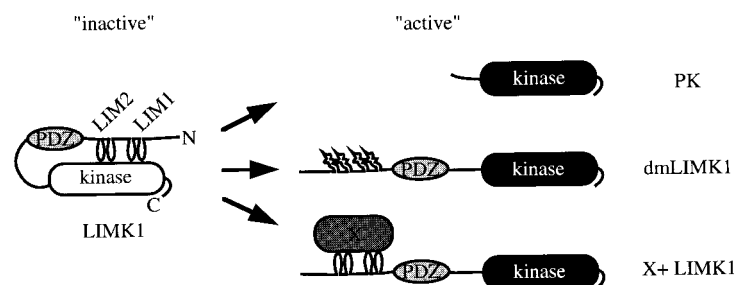
organization were also induced in  $\Delta$ LIM-expressing cells (Figure 5, lower panels), but the pattern of the induced actin assembly significantly differed from the pattern induced by expression of wild-type LIMK1. In  $\Delta$ LIM-expressing cells, large puncta composed of polymerized actin were observed with no detectable stress fibres or any other actin-assembled structure. LIMK1 and  $\Delta$ LIM were enriched in regions of polymerized actin structures. Distinct patterns of actin organization induced by full-length LIMK1 and  $\Delta$ LIM suggest that the LIM domain plays a role in the activity of LIMK1 *in vivo*.

## DISCUSSION

The kinase catalytic activity of many protein kinases is usually suppressed in cells to the basal level and is activated only in response to certain upstream activation stimuli. The extra-catalytic region of the kinase often serves as the domain that negatively regulates the kinase activity, and liberation from its suppressive effect by events such as binding of activators to the extra-catalytic region results in activation of the enzyme [22–24]. In many cases the kinase activity is also regulated by phosphorylation of residues in catalytic and/or extra-catalytic regions by upstream kinases or by autophosphorylation [22–24]. LIMK1 has characteristic structural features containing LIM and PDZ domains in the N-terminal extra-catalytic region. As LIM and PDZ domains are involved in protein–protein interactions, it has been postulated that these domains play a regulatory role in enzyme activity. In the present study we found that deletion or sterically destructive mutation of the LIM domain increased the kinase activity of LIMK1. We also noted that the LIM domain bound directly to the C-terminal kinase fragment and inhibited the kinase activity significantly when added to the C-terminal-kinase core fragment. Taken together these results suggest that the N-terminal LIM domain, by direct interaction with the kinase domain, plays an inhibitory role in the regulation of the kinase activity of LIMK1 (Figure 6). Our previous results showed that only a small population (1–2%) of LIMK1 homodimerizes in cultured cells [25], indicating that the intramolecular rather than intermolecular interaction between the LIM and the kinase domain is usually dominant. The interaction between them may fix the conformation of the kinase domain as an inactive one, or more directly inhibit the access of substrates by masking the kinase catalytic site or substrate-binding site. The LIM domains have been reported to bind various motifs and sequences that are structurally quite different [12–14], but the C-terminal region of LIMK1 has no apparent similarity to any reported LIM-binding motifs.

In the case of LIM-homeodomain protein (LHX) transcription factors, such as Xlim-1 and Isl-1, it was proposed that the LIM domain interacts with the homeodomain within the molecule and that this interaction inhibits the DNA binding and transcription activity of the homeodomain [26,27]. LIM-domain-binding protein/nuclear LIM interactor (LDB1/NLI) was identified as the binding partner of LHXs [28,29]; LDB1/NLI binds to the LIM domains of various LHXs and LIM-only proteins (LMOs) through its C-terminal region and mediates the complex formation of these nuclear LIM proteins by homodimerization of LDB1/NLI through its N-terminal region [30,31]. As LDB1/NLI functionally synergizes with LHXs in target-gene activation, association of LDB1/NLI with the LIM domain seems to release the inhibitory effect of the LIM domain in LHXs. Similar to the case of LHX transcription factors, the kinase activity of LIMK1 may be stimulated by binding of the activator to the LIM domain, as depicted in Figure 6. Although LDB1/NLI has diverse binding specificities with the LIM domains of various LHX- and LMO-family proteins, it did not bind to the LIM domain of LIMK1 or other cytoskeletal LIM-containing proteins [26,27]. The cytoplasmic region of neuregulin (NRGc) was reported to bind to the LIM domain of LIMK1 [17], but in our experiments the binding activity to the LIM domain of LIMK1 was barely detectable and there was no apparent effect on the kinase activity of LIMK1 (K. Ohashi and K. Mizuno, unpublished work). Elucidation of the LIM-binding factor, which we assume to be involved in the activation of LIMK1, will provide the important information for understanding the signalling pathways upstream of LIMK1. We and others recently provided evidence that LIMK1 functions downstream of Rac and mediates Rac-induced actin reorganization [5,6]. However, we observed neither a direct interaction between LIMK1 and Rac nor activation *in vitro* of LIMK1 by Rac [5]. Thus the Rac-induced activation of LIMK1 observed *in vivo* is indirect. Effector proteins for Rac, such as PAK (p21-activated protein kinase), POR1 (partner of Rac 1), POSH (plenty of SH3s), IQGAP (Ras GTPase-activating-protein-related protein with IQ motifs), Sra-1 and WAVE (Wiskott–Aldrich-syndrome-protein family verprolin-homologous protein) [32,33], are candidates that mediate the Rac-induced activation of LIMK1.

As reported [5,6], expression of LIMK1 in cultured cells led to the accumulation of actin filaments (as seen in the upper panels of Figure 5). This is probably due to the function of LIMK1 in phosphorylating and inactivating cofilin [5,6], whose ability to bind to and depolymerize actin filaments is abolished by phosphorylation of Ser-3 [2–4]. Since expression of a kinase-inactive mutant of LIMK1 had no apparent effect on the actin cyto-



**Figure 6** A putative model for regulation of LIMK1 kinase activity

The N-terminal LIM domain usually inhibits the kinase activity of LIMK1 by interaction with the C-terminal kinase domain. Deletion or mutation of the LIM domain activates LIMK1. In living cells, activation may occur by association of the LIM domain with the hypothetical LIM-binding factor, as indicated by X.

skeleton in the cells [5,6], actin-reorganization activity of LIMK1 depends on its kinase catalytic activity. The kinase activity of full-length LIMK1, although it is suppressed to the basal level under the assay conditions *in vitro*, may be enhanced in the cells, either by binding of endogenous activators to the LIM domain or by spontaneous activation due to overexpression.  $\Delta$ LIM, a constitutively active form of LIMK1 with the LIM domain deleted, also induced actin reorganization, but the pattern of accumulation of actin filaments induced by  $\Delta$ LIM (i.e. formation of large puncta of actin aggregates seen in the lower panels in Figure 5) was distinct from that induced by full-length LIMK1. Similar results were shown by Arber et al. [6]. Thus the LIM domain is thought to play a significant role in *in vivo* actin-reorganization activity of LIMK1. The distinct pattern of actin organization in  $\Delta$ LIM-expressing cells may be brought about by exhaustive phosphorylation of cofilin, since  $\Delta$ LIM has the higher and uncontrolled kinase catalytic activity. In addition, such pattern of actin organization may be caused by changes in the subcellular localization of  $\Delta$ LIM, due to the absence of the LIM domain. Whereas the C-terminal kinase domain of LIMK1 has the potential to bind to F-actin [5], the LIM domain may have an additional role in directing LIMK1 to specific cellular loci where it functions.

In this respect, several LIM-containing proteins that associate with actin cytoskeletal components have been identified and are implicated in the regulation of actin reorganization [34–38]. The LIM domains of these proteins are thought to be involved in interactions with actin cytoskeletal components or regulatory factors. For example, cysteine-rich protein 1 (CRP1) binds to  $\alpha$ -actinin through its N-terminal LIM domain [34]; zyxin binds to CRP1 through one of the three LIM domains (LIM1) and binds to  $\alpha$ -actinin and vasodilator-stimulated phosphoprotein (VASP) through the N-terminal non-LIM region [35]; one of the four LIM domains (LIM3) of paxillin is the principal determinant of its focal-adhesion localization [36]. On the other hand, the role of other LIM domains of these proteins or the role of LIM domains of other cytoskeletal LIM proteins, such as abLIM-1 and ALP [37,38], are unknown. To fully comprehend the roles of LIM proteins (including LIMK1) in regulating actin cytoskeletal reconstruction, LIM-binding partners have to be identified. Similar to the case of LIM domains of nuclear LIM proteins (LHX and LMO), which bind to a common LIM-binding partner LDB1/NLI, the LIM domains of cytoskeletal LIM proteins may have another common LIM-binding partner.

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