Structural Features of LIM Kinase That Control Effects on the Actin Cytoskeleton*

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LIM kinase phosphorylates and inactivates the actin binding/dem polymerizing factor cofilin and induces actin cytoskeletal changes. Several unique structural features within LIM kinase were investigated for their roles in regulation of LIM kinase activity. Disruption of the second LIM domain or the PDZ domain or deletion of the entire amino terminus increased activity in vivo measured as increasing aggregation of the actin cytoskeleton. A kinase-deleted alternate splice product was identified and characterized. This alternate splice product and a kinase inactive mutant inhibited LIM kinase in vivo, indicating that the amino terminus suppresses activity of the kinase domain. Mutation of threonine 508 in the activation loop to valine abolished activity whereas replacement with 2 glutamic acid residues resulted in a fully active enzyme. Dephosphorylation of LIM kinase inhibited cofilin phosphorylation. Mutation of the basic insert in the activation loop inhibited activity in vivo, but not in vitro. These results indicate phosphorylation is an essential regulatory feature of LIM kinase and indicate that threonine 508 and the adjacent basic insert sequences of the activation loop are required for this process. A combination of structural features are thus involved in receiving upstream signals that regulate LIM kinase-induced actin cytoskeletal reorganization.

Many protein kinases contain modular domains that regulate catalytic activity, direct localization to specific compartments of the cell, and dictate interactions with other components of signal transduction complexes (1). There are 2 identified LIM kinase family members that each contain 2 amino-terminal LIM domains, a central PDZ domain, and a carboxyl-terminal kinase domain with predominant serine/threonine kinase activity (2–4). During mouse development LIM kinase localizes along actin filament bundles and at adhesion plaques, which results in disruption of cardiomyocyte architecture and dilated cardiomyopathy (5–7). In developing human tissues LIM kinase is also found predominantly in brain where hemizygous deletion of the 7q 11.23 region containing LIM kinase is implicated in the visuo spatial constructive cognition defect in Williams syndrome (8).

Major unanswered questions have been how LIM kinase functions and how it is regulated. Cofilin has been identified as a functionally important substrate for LIM kinase and evidence has been provided that LIM kinase regulates actin dynamics by phosphorylation and inactivation of cofilin (9, 10). Actin-depolymerizing factor/cofilamentous protein (cofilin) which binds to both F-actin and actin monomers (11–13) is essential for depolymerization of actin filaments (14). It binds more tightly to ADP-actin than to ATP-actin and enhances the off rate of actin monomers at the pointed end of fibers (15). At pH 8.0 and above, cofilin depolymerizes actin stoichiometrically (16, 17). This is essential to actin dynamics necessary for cell motility, cytokinesis, and other cell processes (18–20). Cofilin exists in both a phospho and dephospho form, with phosphorylation inhibiting the actin filament severing activity (21, 22). Ser2 is the principal inhibitory phosphorylation site in cofilin and previous studies indicated it was a poor substrate for known kinases including protein kinase C, cyclic AMP-dependent protein kinase, myosin light chain kinase, and CaM kinase II (21). By using dominant negative forms of LIM kinase and constitutively active forms of Rac, Arber et al. (9) and Yang et al. (10) deduced that LIM kinase is the downstream effector of Rac-dependent actin cytoskeleton changes. Because activated Rac did not interact directly with LIM kinase, Rac regulation of LIM kinase activity must involve intermediate biochemical steps.

The kinase domain of LIM kinase contains significant sequence variations compared with other serine/threonine kinases in the ATP-binding site (subdomain VIB) (23), the substrate-binding region of subdomain VIII and in the presence of an 11-amino acid basic insert in the activation loop between subdomains VII and VIII (2–4). Although the sequence of the LIM domains of LIM kinase more closely resemble those of nuclear LIM homeodomain and LIM-only proteins than those of cytoplasmic proteins (24), the LIM domains of LIM kinase do not recognize the nuclear LIM interactor that binds nuclear LIM domains with high affinity (25). The predominant cytoplasmic localization of LIM kinase (2, 4) and its ability to bind actin (10) indicate that it, like many other extranuclear LIM domain containing proteins, functionally associates with the cytoskeleton. Zyxin, cysteine-rich protein and paxillin are localized along actin filament bundles and at adhesion plaques (26, 27). The actin LIM protein is localized to the cytoskeleton via its PDZ domain (28) while cysteine-rich protein 1 binds to α-actinin via its first LIM domain (29). Deletion of muscle LIM protein (cysteine-rich protein 3 or muscle LIM protein), which localizes to actin filaments via LIM domains, results in disruption of cardiomyocyte architecture and dilated cardiomyopathy (30). Some PDZ domains that bind to the consensus SerThrX-Val/Leu/Ile at the carboxyl terminus of target proteins (31) also bind to the cytoskeleton via interaction with α-actinin-2 (28). Other PDZ domain containing proteins also bind to actin via F-actin-binding domains (32, 33). LIM kinase thus contains

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To determine features that regulate LIM kinase we have measured the activity of LIM kinase using transient transfection in COS-7 cells. An in vivo assay of the biochemical activity of LIM kinase is based upon the morphological extent of actin cable dissolution and subsequent aggregation that results from inactivation of cofillin. Mutations were introduced into the LIM, PDZ, and kinase domains to assess their contributions to the activity of LIM kinase that causes actin accumulation in large uncleaved aggregates that were visualized by fluorescently labeled phalloidin binding. Additionally, co-expression of an amino-terminal fragment that corresponds to a naturally occurring splice variant with holo LIM kinase inhibited actin aggregation. These studies indicate that the amino-terminal fragment that contains the LIM and PDZ domains inhibits the catalytic activity of the kinase domain. These studies also indicate that a threonine residue in the catalytic loop, which is a major phosphorylation site in other kinases, is necessary for catalytic activity and that the basic insert in the activation loop contributes to biological activity. The unique structural features of LIM kinase located both outside and within the kinase domain thus control enzyme activity, subcellular distribution, and substrate recognition necessary for regulation of actin dynamics.

EXPERIMENTAL PROCEDURES

Materials—COS-7 and HEK 293 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA). Six-well tissue culture plates and preferred glass coverslips were from Fisher Scientific (Pittsburgh, PA) and cell culture media and serum were purchased from FMC (Rockland, DE). Oligonucleotides used for mutagenesis were made by Operon Technologies (Alameda, CA) and molecular biology enzymes were purchased from New England Biolabs (Beverly, MA). All polymerase chain reaction (PCR)7 products were amplified with Pfu polymerase. Cofilin was a kind gift from Laurent Blanchon and Tom Pollard (Salk Institute, La Jolla, CA).

RT-PCR Cloning and Library Screening—RNA from A431 cells was harvested using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) and reverse transcriptase polymerase chain reaction (RT-PCR) were carried out using the Superscript cDNA Cloning Kit (Stratagene, La Jolla, CA) with random hexamers as the primers for the cDNA synthesis reaction. LIM kinase and dLIM kinase were amplified from the resulting cDNA library using primers with the sequences, 5′-GTCACCTAAGCTCATGAGGTTGACGCTACTTTGT-3′ for the 5′ end, and 5′-GTCATAAAGCTCTACGAGGCTCAGCGCTGGTGGCA- GG-3′ for the 3′ end. The PCR products were amplified with Pfu polymerase, digested with HindIII, and ligated into the HindIII sites in the cloning vector, pBluescriptII-KS (Stratagene). The sequence of the full-length LIM kinase and dLIM kinase were verified by sequencing using Sequenase 2.0 (Amersham, Arlington Heights, IL), with letters corresponding to the one-letter amino acid code. The abbreviations used are: PCR, polymerase chain reaction; nt, nucleotide; RT-PCR, reverse transcriptase-PCR; LIM1, LIM domain 1; LIM2, LIM domain 2; CIP, calf intestinal alkaline phosphatase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase.

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Quickchange mutagenesis kit (Stratagene). A schematic representation of mutant LIM kinases is shown in Fig. 1. Kd1 and Kd3 constructs were made using 5′ primers 5′-GACCTCTCAGGTCGACCTCAGTGACAGCCGCGCGCTCC- CCTGAGGAGGTGCTG-3′ and 5′-GATCCCTCTGCCGGGCGCTCCCTGGG- CAGTCCGGGACCT-3′, respectively, and the 3′ primer 5′-GGCTTCAGT- CAGTCTGAGGACCTC-3′. The constructs were amplified by PCR and ligated into the XhoI and NotI sites of pcDNA-3M, a derivative of pcDNA-3 which contains a luciferase start codon and a hemagglutinin epitope tag (HA-tag) (36). Translation is initiated from the luciferase start codon and places an in-frame HA-tag at the amino terminus of the constructs. All mutations were verified by sequencing.

Cell Lines and Culture Conditions—293 transformed human kidney and COS-7 green monkey kidney cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified 8% CO2 atmosphere at 37 °C. All constructs were cloned into pcDNA-3 (Invitrogen, Carlsbad, CA) except Kd1 and Kd3, which were cloned into pcDNA-3M. DNAs were transfected into COS-7 and 293 cells using calcium phosphate-mediated transfection (37).

Production of Polyclonal Antibodies—A peptide with the sequence KETYRGESGLPAHPEVPD corresponding to the carboxyl-terminal 18 residues and a peptide that corresponds to residues 255–271, KEHPDHTDLGHLGPG, of human LIM kinase were conjugated to keyhole limpet hemocyanin using gluteraldehyde (38). An amino-terminal lysine was included on each peptide to facilitate coupling. The peptide conjugates were used to immunize rabbits or chickens by Lampire Laboratories (Ottsville, PA). Immunoprecipitations were done using the rabbit antibody 5079 directed against the carboxyl-terminal peptide except those involving dLIM kinase where antibody 5078, directed against a different epitope was used. Western blotting was done with the chicken antibody 625 directed to the internal peptide. The Kd3 construct was detected using an anti-HA monoclonal antibody (Berkeley Antibody Co., Berkeley, CA).

Immunochemistry—Cells used for immunocytochemistry were plated onto preferred glass coverslips and harvested 60 h after transfection. Cells were fixed with a 4% paraformaldehyde in 1 × phosphate-buffered saline for 25 min at room temperature. To determine LIM kinase expression, cells were incubated 2–3 h with a 1:1,250 dilution of anti-LIM kinase rabbit antibody 5079 in buffer containing 0.1% Triton X-100, 0.024% saponin, and 2% bovine serum albumin. To visualize LIM kinase and to stain for actin filaments, cells were incubated for 2 h in buffer containing goat anti-rabbit Texas Red-conjugated secondary antibody and Oregon Green 488-labeled phalloidin (Molecular Probes, Eugene, OR). Cells were then equilibrated and mounted with Slow Fade mounting media. Pictures were taken with a Zeiss Axiophot microscope with an attached Hamamatsu color chilled CCD camera using ×40 and 60 objectives.

Immunoprecipitation and Kinase Assays—Transiently transfected 293 cells were grown for 72 h and harvested by washing cells off the plates with phosphate-buffered saline and resuspending in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 3% glycerol, 2% Triton X-100, 50 μM benzamidine, 2 μM aprotinin, 2 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml phenanthrol) and incubated on ice for 30 min. Cleared lysate was presorbed with preimmune serum and protein A-Sepharose. LIM kinase was then immunoprecipitated using the 5079 anti-LIM kinase antibody and protein A-
Regulation of LIM Kinase Activity

Expression

LIMK WT-LIMK Actin Aggregation

A

B

C

D

E

F

G

H

I

J

D460N

Fig. 2. LIM kinase induces an actin aggregation phenotype that is dependent on kinase activity. COS-7 cells were transfected with 1 μg of either WT LIM kinase (A–H) or mutant kinase-inactive LIM kinase D460N (I–J). Cells were transfected, harvested, and stained with anti-LIM kinase antibodies (A, C, E, G, and I) or phallolin (B, D, F, H, and J). The extent of changes in the actin cytoskeleton are indicated by the scale on the right and the intensity of LIM kinase staining is indicated on the left. The scale bar equals 20 μm, for all figures. The same magnification was used in panels A, B, G–J, and in panels C–F.

A mutation of the predicted catalytic base that changes Asp to Asn (D460N) is reported to abolish LIM kinase activity (6). Even high levels of expression of a D460N mutant LIM kinase gave no change in the actin cytoskeleton compared with untransfected cells (Fig. 2, I and J). In vitro kinase assays confirmed that D460N LIM kinase was devoid of catalytic activity (see Fig. 10). The changes in the actin cytoskeleton were thus dependent on the kinase activity of LIM kinase.

Effect of Mutations of the LIM and PDZ Domains on LIM Kinase-induced Changes in the Actin Cytoskeleton.—To assess the role of the LIM and PDZ domains on LIM kinase function, mutations were introduced which were designed to disrupt each of these domains. The structure of LIM domains is dependent on two Zn2+ atoms that are coordinated tetrahedrally in amino- and carboxyl-terminal liganding modules (39). Metal binding is essential for protein structure and renaturation studies indicate that binding is sequential (40). The conserved Cys residue of each LIM domain of LIM kinase was mutated to Ser to disrupt metal coordination and LIM domain structure (C25S for the first (LIM1) and C84S for the second (LIM2) LIM domain). Mutations in the PDZ domain were based on the crystal structure of the third PDZ domain of PSD-95 complexed to the carboxyl terminus of the potassium channel (41). The signature sequence Gly-Leu-Gly-Phe that constitutes the protein binding loop of PDZ domains is 177Gly-Leu-Ser-Val in LIM kinase. To disrupt target binding, the LIM kinase PDZ

RESULTS

Effects of LIM Kinase on the Actin Cytoskeleton.—To investigate the functional consequences of mutations in LIM kinase, an in vitro assay was developed based on the effects of transiently transfected LIM kinase on the actin cytoskeleton in COS-7 cells. Expression of LIM kinase was assessed using a rabbit polyclonal anti-peptide antibody directed to the carboxy terminus and effects on the actin cytoskeleton were assessed using fluorescently labeled phalloidin. Fig. 2 shows the range of changes observed in the actin cytoskeleton; protein expression levels were based on the intensity of immunofluorescence and were qualitatively proportional to the extent of changes in the actin cytoskeleton indicated in the panels on the right. At low levels of LIM kinase expression there was a decrease in the extent of actin cables visualized and enhanced membrane staining (Fig. 2, A, C, E, G, and I) or mutant kinase-inactive LIM kinase (Fig. 2, B, D, F, H, and J). The extent of changes in the actin cytoskeleton are indicated by the scale on the right and the intensity of LIM kinase staining is indicated on the left. The scale bar equals 20 μm, for all figures. The same magnification was used in panels A, B, G–J, and in panels C–F.

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domain 177Gly-Leu was mutated to Glu-Ala (G177E/L178A).

These mutations are predicted to disrupt both alignment of backbone interactions and the hydrophobic pocket necessary for protein binding (41). Mutation of the corresponding residues in the PDZ domain of Enigma, which also contains a variant sequence at this position, abolished target recognition.2

As shown in Fig. 3, A and B, mutation of LIM1 did not affect the ability of LIM kinase to induce actin aggregation. Aggregates of actin were observed comparable to those observed with similar levels of WT LIM kinase (see Fig. 2D). Mutation of LIM2 and the PDZ domain increased the ability of LIM kinase to induce actin aggregation (Fig. 3, C-F). At low levels of G177E/L178A LIM kinase expression, phenotypic changes scored as 3+ to 4+ were seen whereas comparable levels of holokinase expression gave actin cytoskeleton changes scored as 1+ to 2+. As shown in the left panels, mutations in the LIM and PDZ domains did not affect subcellular localization as these mutant LIM kinases remained outside the nucleus. These data indicate that LIM and PDZ domains are not necessary for biological responses to LIM kinase-induced actin aggregation; however, increased activity upon mutational inactivation of the LIM2 and PDZ domains suggests these suppress LIM kinase activity in the holoenzyme structure.

Although transfection of various LIM kinase mutants resulted in equivalent average protein expression per dish (see Fig. 11), expression in individual cells within the population varied as shown in Fig. 2. To assess the relative intrinsic activities of each form of the LIM kinase, the distribution of induced changes in the actin cytoskeleton in a population of cells expressing each mutant were scored and presented as described by Arber and co-workers (9). Fig. 4 shows that actin cytoskeletal changes induced by WT-LIM kinase in COS-7 cells were primarily those indicated by 3+ and 2+ qualitative scores in Fig. 2. Mutational inactivation of LIM2 and the PDZ domain shifted the distribution toward the more severe actin aggregation phenotype indicated as 4+ in Fig. 2H whereas the D460N LIM kinase had little effect on the actin cytoskeleton relative to mock transfected cells.

**Expression of the Kinase Domain of LIM Kinase Is Sufficient to Induce Actin Aggregation**—The observation that disruption of the PDZ domain and the second LIM domain enhanced the *in vivo* activity of LIM kinase suggested that the kinase domain was sufficient to induce changes in the actin cytoskeleton. Two kinase constructs containing the kinase domain of LIM kinase were made and expressed in COS-7 cells (Fig. 1). Kd1 was composed of the conserved catalytic core of the kinase domain from the glycine-rich loop to the end of LIM kinase (residues 346–647). This construct, which includes all of the conserved residues in the catalytic core, does not include the α helix, which runs down the back of the large and small lobes of cAMP-dependent protein kinase and is important for stability and catalytic activity of that enzyme (42). The second construct, Kd3, which is composed of the entire kinase domain, included the α helix (residues 302–647). This construct corresponds to a naturally occurring splice variant of LIM kinase that is found in testis (43). Even high levels of expression of Kd1 failed to induce changes in the actin cytoskeleton (Fig. 5, A and B). The punctate perinuclear staining suggested that Kd1 may not be folded and processed normally. In contrast, Kd3 was extremely active, inducing a strong aggregation phenotype (Fig. 5, C and D; Fig. 4). The complete kinase domain with the α helix is thus required for biological effects on the actin cytoskeleton.

The enhanced activity of Kd3 supports the idea that the amino terminus of LIM kinase suppresses activity of the holoenzyme.

**A Splice Variant of LIM Kinase Results in a Kinase-deleted Form**—During cloning of the full-length LIM kinase transcript from an A431 cell cDNA library, a splice variant was found that was identical to the LIM kinase sequence with the exception of a 61-base pair deletion upstream of the region coding for the kinase domain. The deletion, from nt 977 to 1037 in the cDNA relative to the start codon (Fig. 6A), is similar in size to, but differs from, the deletion in the kinase core of LIM kinase reported previously (2). Analysis of the genomic sequence (8) showed that the deletion was the result of differential splicing at the 3’ end of intron 7 which joins with the middle of exon 8.

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### Footnote

1 P. M. Guy, D. A. Kenny, and G. N. Gill, submitted for publication.
Expression and aggregation were graded as indicated. Expression and aggregation were graded as indicated.

at nt 23116 instead of at the 5' end of exon 8, at nt 23055 as occurs with full-length LIM kinase (Fig. 6B). The deletion, located in the sequence between the PDZ domain and the kinase domain, causes a frameshift at amino acid 294 adding 12 amino acids not found in the full-length protein and resulting in a premature truncation at amino acid 305 (Fig. 6C). This truncation deletes the entire kinase domain, resulting in a catalytically inactive protein, termed deleted LIM kinase (dLIM kinase). To determine if the amino-terminal splice variant dLIM kinase was found elsewhere, 18 cDNA libraries, either commercially available or made from cultured cells, were screened using RT-PCR and primers that allow discrimination between dLIM kinase and LIM kinase. Fig. 6D shows that full-length LIM kinase was found in all samples analyzed (upper band), while dLIM kinase was found in 9 of the 18 analyzed (lower band). In vitro transcription/translation reactions showed that dLIM kinase encoded a 32-kDa protein (data not shown). The truncated protein failed to induce actin bundling in COS-7 cells, and was expressed primarily in the cytoplasm with accumulation in the perinuclear region (Fig. 6E).

The Kinase-inactive D460N LIM Kinase and the dLIM Kinase Splice Variant Inhibit LIM Kinase Activity—The effect of the kinase-inactive D460N mutant on wild type LIM kinase activity was investigated by co-transfecting expression constructs for both proteins into COS-7 cells. As shown in Fig. 7, A and B, D460N LIM kinase significantly blocked the aggregation of actin induced by wild-type LIM kinase. The inhibitory effects of D460N LIM kinase on the actin phenotypes induced by WT LIM kinase are evident in the distributions shown in Fig. 4. Although some effects of LIM kinase on the actin cytoskeleton persisted, the D460N mutant largely inhibited the biological effects not only of wild-type LIM kinase, but also of LIM kinase containing mutations in LIM1 (C25S), LIM2 (C84S), and the PDZ domain (G177E/L178A) (data not shown).

Hiraoka and co-workers (44) reported that when HA-tagged LIM kinase 1 and untagged wild-type protein were coexpressed in COS cells, the two co-immunoprecipitated. Use of GST fusions of fragments of LIM kinase indicated that the self-association of LIM kinase involved interaction of the amino terminus with the carboxyl terminus. The inhibitory effect of D460N on wild-type LIM kinase activity in vivo is compatible with the dominant negative effect being due to the postulated dimerization of the two proteins. Because D460N LIM kinase inhibited the function of LIM kinase that contained mutations in the LIM and PDZ domains it was uncertain which regions were responsible for inhibition. To address this question, we coexpressed the amino-terminal splice variant that lacks the kinase domain and the inactive kinase fragment Kd1, with both wild type LIM kinase and the active kinase fragment Kd3. dLIM kinase inhibited the actin aggregating effects of LIM kinase whereas the inactive kinase fragment Kd1 did not (Fig. 7, C-F). Moreover, dLIM kinase inhibited the activity of the Kd3 kinase domain-only construct whereas Kd1 was without effect (Fig. 7, I-L). In contrast the kinase inactive D460N LIM kinase which inhibited holo LIM kinase did not inhibit Kd3 (Fig. 7, G and H).

The finding that the amino-terminal splice variant dLIM kinase inhibited the activity of Kd3 domain provides functional support for the model of Hiraoka et al. (44) in which LIM kinase monomers self-associate in an antiparallel fashion. The present data indicates that it is the amino terminus which suppresses the kinase activity of the dimeric enzyme. While Hiraoka and co-workers (44) found the LIM domains to be necessary for self-association, the present data indicate that mutation of either LIM domain alone does not abolish interac-
tion as assessed by in vivo activity. An amino-terminal fragment consisting of the 2 LIM domains alone also inhibited LIM kinase activity (data not shown), suggesting that both LIM domains function in this manner. The failure of D460N LIM kinase to inhibit Kd3 suggests that self-association of holoenzyme monomers in which both partners contribute amino- and carboxyl-terminal interaction domains is stronger than association of fragments.

**Mutations in the Activation Loop of the Catalytic Core Affect LIM Kinase Activity**—Kinases are regulated by both modular domains and phosphorylation (1, 45). Phosphorylation of a conserved Thr residue in the activation or T loop of several kinases including cAMP-dependent protein kinase, cyclin-dependent kinase 2, and MAP kinase enhances catalytic efficiency (46, 47). MAP kinase is also phosphorylated on Tyr in the sequence Thr-Glu-Tyr in the activation loop and phosphorylation of both sites is required for maximal activity (48, 49). The analogous activation loop Thr residue in LIM kinase, Thr<sup>508</sup>, is adjacent to a Tyr residue, Tyr<sup>507</sup>. Proschel et al. (6) reported that a GST kinase domain LIM kinase fusion protein exhibited in vitro autophosphorylation on Ser and Tyr residues with only trace amounts of phosphate on Thr.

To evaluate the requirement for Thr<sup>508</sup> and Tyr<sup>507</sup> as potential regulatory phosphorylation sites in LIM kinase, Thr<sup>508</sup> was mutated to Val (T508V) and Glu (T508E) and activities were assayed. T508V abolished the ability of LIM kinase to induce changes in the actin cytoskeleton in vivo (Figs. 4 and 8, A and B). This loss of enzyme activity suggests that phosphorylation of Thr<sup>508</sup> in the activation loop is essential for enzymatic activity. The T508V LIM kinase also interfered with the actin cytoskeleton changes induced by WT LIM kinase (Fig. 4). Changing Thr508 to Glu, which is reported to constitutively activate protein kinase C by mimicking a phosphorylated Thr (50) also resulted in an enzyme that did not exhibit LIM kinase activity as assessed by changes in the actin cytoskeleton (Figs. 4 and 8, C and D).

To further investigate the potential effects of phosphorylation of Thr<sup>508</sup>, this residue was mutated in the highly active kinase-only construct Kd3. While Kd3 induced severe actin aggregation (Fig. 9, panels A and B), substitution of Glu for Thr at residue 508 failed to induce actin aggregation in COS-7 cells (Fig. 9, panels C and D), comparable to lack of effects of the kinase inactive Kd3-D460N mutant (Fig. 9, panels G and H). In contrast, substitution of 2 Glu residues whose charge may more closely resemble a phosphate on Thr (Kd3-T508EE) resulted in full activity comparable to that of Kd3 (Figs. 4 and 9, E and F). These findings resemble studies of Mek1 where substitution of an Asp or Glu residue for 1 serine phosphorylation site partially activated the enzyme and substitution of 2 acidic residues for both serine phosphorylation sites resulted in a fully active enzyme (51). Together, these results suggest that phosphorylation of Thr<sup>508</sup> is required for LIM kinase activity. In contrast changing T507F did not reduce LIM kinase activity.

**Fig. 8.** Thr<sup>508</sup> is necessary for LIM kinase effects on the actin cytoskeleton. COS-7 cells were transfected with 1 μg of T508V LIM kinase (A and B), T508E LIM kinase (C and D), or Y507F LIM kinase (E and F). Cells were stained for LIM kinase (left panels) and actin (right panels).
Regulation of LIM Kinase Activity

**Fig. 9.** Two glutamic acid residues in the activation loop activate LIM kinase. COS-7 cells were transfected with Kd3 (A and B), Kd3 containing a single Glu replacement of Thr (T508E) (C and D), Kd3 containing 2 Glu residues in this area (T508EE) (E and F) or the kinase-inactive mutation D460N in Kd3 (G and H). Cells were stained for LIM kinase (left panels) and actin (right panels).

Fig. 10. The basic insert sequence affects the actin aggregating activity of LIM kinase. COS-7 cells were transfected with 1 μg of either kinase insert-deleted LIM kinase (A and B), the 3 amino acid kinase insertion mutation (C and D), or Kd3 containing the kinase insert mutation (E and F). Cells were stained for LIM kinase (left panels) and actin (right panels).

on the actin cytoskeleton (Fig. 8, panel E). Thus, Thr\(^{508}\) is likely an essential phosphorylation site in LIM kinase whereas Tyr\(^{297}\) is not. The failure to autophosphorylate on Thr \textit{in vitro} (6) and the lack of kinase activity of bacterially expressed Kd3 (data not shown) suggest Thr\(^{508}\) is phosphorylated \textit{in trans} by a distinct kinase.

The activation loop located between subdomains VII and VIII in the catalytic core has been shown to influence substrate recognition in MAPK (52). Both LIM kinases 1 and 2 contain a unique highly basic 11 amino acid insert in the activation loop that may function in recognition of the unusual amino-terminal phosphorylation site on cofilin. Alternatively, the basic residues may provide the substrate determinants for Thr\(^{508}\) analogous to those of other substrates for kinases such as cAMP-dependent protein kinase and protein kinase C (53, 54). To investigate the function of this insert, 2 mutants were constructed: in one, the kinase insert was deleted (KI-del.) and in the other, the basic residues Arg-Lys-Lys were mutated to Gly-Ala-Ala (KI-mut) (Fig. 1). Both mutants failed to induce significant actin aggregation in COS-7 cells (Fig. 10, A-D). Activity was clearly reduced compared with the wild-type enzyme (Fig. 10, E and F) as even high levels of expression of these mutant forms of LIM kinase resulted in no more than 2 times changes in the actin cytoskeleton. No change in subcellular localization was noted by mutation or deletion of the kinase insert region.

**Mutations in LIM kinase Affect Phosphorylation of Cofilin in Vitro**—Immunoprecipitated LIM kinase-catalyzed phosphorylation of cofilin \textit{in vitro}. As shown in Fig. 11A, both wild-type and Kd3 LIM kinase catalyzed phosphorylation of cofilin \textit{in vitro}. LIM kinase catalyzed phosphorylation of myelin basic protein to a low stoichiometry whereas cAMP-dependent protein kinase and protein kinase C efficiently catalyzed phosphorylation of MBP but not cofilin (data not shown).

To assess the effects of phosphorylation of LIM kinase on kinase activity, immunoprecipitates of wild-type and Kd3 LIM kinase were treated with CIP to remove any phosphate groups and cofilin phosphorylation was measured. As shown in Fig. 11A, CIP treatment abolished the activity of both forms of LIM kinase. Mutation of Thr\(^{508}\) to either Val or Glu markedly reduced kinase activity \textit{in vitro} in agreement with loss of the effects of these enzymes on the actin cytoskeleton \textit{in vivo} (Fig. 11B and Table I). However, substitution of 2 Glu residues for the single Thr (Kd3-T508EE) yielded a fully active enzyme (Fig. 11B and Table I). Enzyme activity \textit{in vitro} thus paralleled effects \textit{in vivo} indicating that Thr\(^{508}\) is a phosphorylation site that is essential for LIM kinase activity.

Mutations in the LIM and PDZ domains did not change the ability of LIM kinase to catalyze cofilin phosphorylation \textit{in vitro} (Fig. 11A and Table I). Similarly, mutations in the kinase insert retained significant activity (Fig. 11B). In more quantitative solution assays of LIM kinase-catalyzed cofilin phosphorylation the kinase insert mutant was also equal to that of the WT enzyme (Table I). All forms of LIM kinase that phosphorylated cofilin to detectable levels also exhibited self-phosphorylation (Fig. 11B). These results suggest that \textit{in vitro} kinase assays reflect the basal activity of LIM kinase. In the absence of the kinase insert LIM kinase cannot be activated \textit{in vivo}, resulting in the observed marked reduction in actin aggregation compared with WT LIM kinase. The insert in the activation loop along with Thr\(^{508}\) is thus necessary for full \textit{in vivo} activity. The \(K_m\) of LIM kinase for cofilin is 7.6 μM compatible with the intracellular concentration of cofilin (Fig. 11C).

To demonstrate that the phosphorylation seen \textit{in vitro} is due to LIM kinase phosphorylation of cofilin at serine 3, a series of cofilin mutants were assessed. Fig. 11D shows that wild-type cofilin, actophorin, and actophorin mutated at serine 84, but not cofilin mutated at serine 3, were phosphorylated by LIM kinase \textit{in vitro}. This demonstrates that LIM kinase specifically
phosphorylates serine 3, in agreement with previously published data (9, 10). Under saturating conditions 1 mol of phosphate was incorporated per mol of cofilin. To demonstrate that the amino terminus of LIM kinase specifically inhibits the kinase activity of LIM kinase, purified Kd3 LIM kinase was incubated without or with a GST fusion to the dLIM kinase and in vitro kinase activity measured. As seen in Fig. 11E, GST-dLIM kinase but not GST inhibited LIM kinase catalyzed cofilin phosphorylation.

**DISCUSSION**

The actin cytoskeleton is a dynamic structure in which the rates of polymerization and depolymerization of actin control cell motility, cell division, and the formation of specialized structures. The actin-binding protein, cofilin, is an important regulator of this process, mediating cleavage and disassembly of actin filaments (14). Phosphorylation of cofilin at Ser3 in the amino terminus inactivates the actin cleaving/depolymerizing activity of this protein (55). Phosphorylation and dephosphorylation of cofilin thus provides an important control point for dynamic changes in the extent of actin polymerization and consequent function. Arber et al. (9) and Yang et al. (10) provided evidence that LIM kinase phosphorylates and inactivates cofilin. In co-transfection and microinjection experiments LIM kinase blocked the effects of cofilin, but not those of a mutant cofilin containing an Ala replacement of the Ser3 phosphorylation site. A kinase-inactive form of LIM kinase lacked activity but blocked the effects of LIM kinase. Importantly, both Arber et al. (9) and Yang et al. (10) reported that the LIM kinase mediated phosphorylation of cofilin and resultant cytoskeletal changes were enhanced by the constitutively active V12 mutant of Rac and reduced by the dominant negative N17 mutant of Rac. These findings support a model in which Rac activates

| TABLE I  
| Cofilin phosphorylation by various forms of soluble LIM kinase |
| Cytosols containing the indicated forms of LIM kinase expressed in HEK 293 cells were incubated with 20 μM cofilin in kinase reaction mixtures for 6 min at 30 °C and 32P incorporation into cofilin was measured as described under “Experimental Procedures.” Activities were corrected for the amount of enzyme used based on quantitation via Western blotting. |

| LIM kinase Activity (pmol of [32P]Phosphate incorporated) | Relative activity* |
|-----------------------------------------------------------|-------------------|
| WT | 34.8 ± 3.2 | 1.0 |
| C25S | 32.1 ± 2.8 | 0.9 |
| C84S | 33.1 ± 3.0 | 1.0 |
| G177E/L178A | 38.2 ± 2.5 | 1.1 |
| D460N | 6.0 | 0.0 |
| T508V | 11.8 ± 4.2 | 0.3 |
| Y507F | 33.3 ± 3.6 | 1.0 |
| Kd3 | 82.6 ± 9.2 | 2.4 |
| Kd3-del | 81 ± 7.5 | 2.3 |
| Kd3-EE | 114.9 ± 10.8 | 3.3 |

* The activity of wild-type LIM kinase was set to 1.0 for comparison with various mutant LIM kinases.

Fig. 11. In vitro phosphorylation of cofilin by wild-type and mutant LIM kinase. All DNAs were transfected into HEK 293 cells and lysates were used in in vitro kinase assays and Western blotting as described under “Experimental Procedures.” A, left: LIM kinase inactivation by dephosphorylation. Immunoprecipitated LIM kinase and Kd3 were incubated without or with CIP, washed, and in vitro kinase activity assayed using cofilin and [γ-32P]ATP as substrates. An autoradiogram of 32P-labeled cofilin (upper panel) and a Western blot of immunoprecipitated LIM kinase and Kd3 (lower panel) are shown. A, right: effects of mutations in the amino terminus of LIM kinase on phosphorylation of cofilin. Immunoprecipitated LIM kinase mutants were assayed for in vitro kinase activity (upper panel) and blotted for protein amount (lower panel). B, effects of mutations within the kinase domain on activity. Various forms of LIM kinase were immunoprecipitated and assayed for phosphorylation of cofilin (upper panel). Western blotting was used to adjust the amount of enzyme used in enzyme assays (lower two panels). C, determination of Km for cofilin. The indicated concentrations of cofilin were added to solution assays containing WT and mutant cofilin (serine 3 to alanine, S3A) and WT and mutant actophorin (serine 84 to alanine, S84A). Two μg of substrate were used in each reaction. D, effect of dLIM kinase on LIM kinase activity. Reactions contained soluble WT LIM kinase alone or with a 5-fold molar excess of GST dLIM kinase or GST.
LIM kinase which phosphorylates and inactivates cofilin resulting in decreased rates of actin depolymerization. The mechanisms through which Rac regulates LIM kinase activity are unknown but do not involve direct interactions between these two proteins (10).

The present studies confirm cofilin phosphorylation by LIM kinase and demonstrate that in vivo LIM kinase causes changes in actin cables with progressive accumulation of large aggregates of actin consistent with loss of cofilin function. This biological “read out” was used to assess the contribution of various regions of LIM kinase to activity and to compare these results to cofilin phosphorylation in vitro.

While the structure of LIM kinase is unique in having 2 amino-terminal LIM domains and a PDZ domain, it resembles other kinases such as Src whose activity is regulated by modular protein domains located outside the catalytic kinase core (56, 57). Both LIM and PDZ domains function in protein-protein interactions as do the SH2 and SH3 domains in Src (58–60). While the LIM and PDZ domains of LIM kinase likely recognize proteins important in the overall biological function of this protein, the present data indicate these domains also regulate the kinase activity of LIM kinase. Mutation of L1M1 had no detectable effect on LIM kinase activity but mutational inactivation of the second LIM domain and of the PDZ domain enhanced biological activity of LIM kinase in vivo, suggesting these domains directly or indirectly restrict activity. The observation that a kinase-only construct Kd3 also exhibited enhanced biological activity supported this idea. The kinase-inactive D460N LIM kinase acted in a dominant interfering manner consistent with in vitro studies showing self-association (44). The finding that a naturally occurring splice variant of LIM kinase that consists of the amino terminus only (LIM plus PDZ domains) inhibited not only the wild-type but also the kinase-domain only Kd3 mutant suggests that the self-association between the amino- and carboxy-terminal portions observed in vitro also occurs in vivo. These data support a model of LIM kinase in which the amino terminus interacts with the kinase domain to suppress activity. In addition to the splice variant identified in this article, one LIM kinase-1 and four LIM kinase 2 splice variants have been identified (2, 6, 43). Three of these variants contain deletions that disrupt the kinase domain while one contains only a kinase domain and is expressed only in testis. The ability of dLIM kinase to inhibit Kd3 suggests that these kinase inactive splice variants may, depending on levels of expression, function as naturally occurring inhibitors of LIM kinase.

Several motifs within the kinase domain appear to control kinase activity. The kinase domain contains both Thr and Tyr residues in the activation loop as well as a distinct basic 11-amino acid insertion. The present data indicate that Thr508 is an essential phosphorylation site but Tyr507 is not. A Val substitution at residue 508 abolished actin aggregating activity and a negatively charged Glu that was predicted to partially mimic phosphorylated Thr similarly resulted in loss of activity. However, placement of 2 Glu residues in this position resulted in a fully active enzyme both in vivo and in vitro. Phosphorylation of Thr in the activation loop of several Ser/Thr kinases is essential for catalytic activity (61). In the MAPK signal transduction pathway, both MAPK kinase (Mek) and MAPK are regulated by dual phosphorylations in the activation loop. Mek1 is activated by phosphorylation of 2 nearby Ser residues and replacement of both with Asp is necessary for full activity (51). Mek activation of MAPK requires phosphorylation of both a Tyr and Thr residue in the activation loop (49). However, a kinase and CDK are phosphorylated on a single Thr residue in this loop (45, 46). While additional phosphorylations may regulate LIM kinase, that of Thr508 is essential. The requirement for 2 Glu residues for activity may be due to interactions with the adjacent basic insertion region. Because bacterially expressed LIM kinase did not exhibit self-phosphorylation on Thr (and had very low catalytic activity) (6) phosphorylation of Thr508 is predicted to occur in trans. The sequence of the Thr508 site in LIM kinase, 504-Lys-Lys-Arg-Tyr-Thr-Val-Val, clearly differs from the phosphorylation site in cofilin (2, 11) making autophosphorylation unlikely. LIM kinase contains the determinants in the active site that are characteristic of protein kinases which are activated by phosphorylation (61). Thus, upstream signals may be transmitted to LIM kinase both via the amino-terminal LIM and PDZ domains and via regulation of a kinase that phosphorylates LIM kinase at Thr508 to activate the enzyme. Protein kinases that are regulated by Rac (for review, see Ref. 62) are candidates to be involved in the signal transduction pathway linking Rac to LIM kinase activation and consequent cytoskeleton changes.

The basic insert in the activation loop which precedes Thr508 likely provides important substrate determinants for phosphorylation at this site. Deletion or mutation of basic residues in this insertion largely abolished LIM kinase activity in vivo. However, the KI deletion did not decrease LIM kinase-catalyzed phosphorylation of cofilin in vitro. These results suggest that while the insert is not required for basal LIM kinase activity, it is necessary for activation of the enzyme in vivo.

LIM kinase thus contains a number of regulatory features consistent with a pivotal role in control of dynamics of the actin cytoskeleton. Relief of inhibitory constraints imposed by amino-terminal LIM and PDZ regulatory domains and phosphorylation of an activation loop Thr are consistent with the general structural features that regulate the activity of other protein kinases and are proposed to be the targets for receipt of upstream signaling information.

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