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David E. Edwards 6/9/99
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Introduction

Primary tumors of the breast are not life threatening. Metastasis of primary tumor cells to essential organs pose the main threat to life for people with breast cancer. Metastasis, the process of cells egressing from the primary tumor site and infiltrating distal tissues, is dependant on cell movement and shape, which in turn are controlled by the cytoskeleton. While cells undergo constant reorganization, the upstream processes that regulate cell shape and movement are not well characterized. The Rho family of GTPases, which include Rho, Rac and Cdc42, were shown to be a common pathway by which signal transductions cascades regulate cell shape and motility. Recently, LIM kinase was shown to regulate the actin cytoskeleton via phosphorylation and inactivation of the actin depolymerizing protein cofilin and be downstream of Rac. Very little is known about the mechanisms that regulate this activity *in vivo*. The research supported by this grant investigates autoregulation of LIM kinase activity *in vivo* (J. Biol. Chem. **274**, 11352-11361) and *in vitro* and *in vivo* regulation of LIM kinase by Rac/Cdc42 and their effector Pak (Submitted, Nature Cell Biology).

Body

LIM kinase is a recently discovered enzyme shown to be a member of the kinase superfamily of proteins and to contain several possible regulatory protein:protein interaction domains in its amino terminus. This kinase was shown to phosphorylate the actin depolymerizing protein cofilin and to be downstream of the Rac GTPase (Arber et al., 1998). However, little was known about the regulation of LIM kinase and the role that LIM kinase had in regulation of the actin cytoskeleton. The goal of this work was, originally, to identify proteins that interacted with and regulated LIM kinase within the cell. The combination of protein:protein interaction domains and the catalytic kinase domain, suggests that regulation of LIM kinase activation is a complex process. The data presented here describes the role that the amino terminal regulatory domains and unique sequences within the kinase domain play in regulating LIM kinase function *in vivo* (Manuscript attached (J. Biol. Chem. **274**, 11352-11361)) and describe the mechanisms involved in upstream regulation of LIM kinase by the Rho family of GTPases *in vivo*. (Submitted manuscript attached).

Regulation by the Amino Terminus

While mutation of the first LIM domain had no effect on LIM kinase's actin bundling activity or *in vitro* kinase activity, and mutation of the second LIM domain only marginally increased the ability of LIM kinase to induce actin bundles, mutation of the PDZ domain greatly increased the activity of LIM kinase *in vivo*. These data suggest that one of the roles of the PDZ domain is to regulate the kinase activity of LIM kinase. This is supported by the fact that the kinase domain of LIM kinase, when expressed

alone, is 2.5 fold more active than full length LIM kinase *in vitro* and much more active *in vivo*. The GL177EA mutant in the PDZ domain of LIM kinase was designed to disrupt the binding of a peptide ligand to the PDZ domain, but to retain the overall structure of the PDZ domain. While mutation of the PDZ domain had little effect on the kinase activity of LIM kinase *in vitro*, a very large change could be seen in the ability of LIM kinase to bundle actin *in vivo*. This suggests that the PDZ domain binding inhibits the kinase activity of LIM kinase *in vivo*, either by binding to an inhibitor protein or by changing the subcellular localization of LIM kinase and not by binding to the kinase domain. The increased activation of LIM kinase *in vivo* but not *in vitro*, suggests that these mutations are the result of an inhibition of the PDZ domain's function *in vivo* and not a result of a generalized, non specific change in the structure of LIM kinase that results in activation of the kinase. The PDZ domain could also function in other ways. Data from Yang and co-workers (Yang et al., 1998) suggests that the PDZ domain contains two nuclear export signals that may regulate localization of LIM kinase to the nucleus. The large increase in kinase activity from deletion of the amino terminus that is not attributable to the PDZ domain suggests that the LIM domains have a significant role in LIM kinase regulation *in vivo* and *in vitro*. Interestingly, the lack of a large impact on the kinase activity by mutation of either LIM 1 or LIM 2, suggests that the LIM domains may be redundant, so that disruption of either domain would not give a phenotype, but disruption of both would. Supporting this conclusion are the facts that a construct of just the amino terminal LIM domains was able to inhibit kinase activity *in vivo* (Data not shown) and the LIM domains bind the Kinase domain *in vivo* and *in vitro* (Hiraoka et al., 1996). Besides binding the kinase domain, the LIM domains have also been shown to

bind to PKC (Kuroda et al., 1996) and transmembrane neuregulins (Wang et al., 1998), though the functional relevance of these interactions is not known.

The splice variant identified in this work, which consists of the amino terminal domains of LIM kinase was shown to inhibit the function of LIM kinase *in vivo* and *in vitro*. These data and the variable expression levels in different tissues and cell lines suggests that this splice variant may regulate LIM kinase activity *in vivo*. The presence of other splice variants, all of which contain complete LIM2 and PDZ domains, suggests that these domains are required for interaction with and regulation of the full length protein *in vivo*. This hypothesis is supported by the data provided here that shows that disruption of these two domains, and not LIM1, which is partially deleted in many of these splice variants, affects LIM kinase activity *in vivo*.

The complete role of the amino terminus of LIM kinase function is still unknown. While the mutations to the PDZ domain activated LIM kinase function *in vivo*, they had little affect on LIM kinase activity *in vitro*. This suggests that the increase in basal LIM kinase activity seen with deletion of the amino terminus is not just the result of deletion of the PDZ domain. While mutation of any one of the LIM or PDZ domains in LIM kinase was not enough to activate LIM kinase activity *in vitro*, perhaps the combination of two or three of these domain mutations would lead to *in vitro* activation. The effect of mutations to the LIM and PDZ domains must be tested in the context of purified, full length LIM kinase *in vitro*, before their role in LIM kinase autoregulation can be fully understood. The autoregulation of LIM kinase by the amino terminus may also be due to sequences outside of the LIM and PDZ domains but within the amino terminus or may be

within these domains, but not dependent on the protein:protein interaction activity of these regions.

Role of Thr508 and the Kinase Insert

In the second chapter, we report that mutations to threonine 508 in the activation loop of LIM kinase had a dramatic effect on the ability of LIM kinase to induce changes in the actin cytoskeleton. While the T508V and T508E mutations were inactive *in vivo* and *in vitro*, the Y507F mutation retained full activity, suggesting that this region was not hypersensitive to structural changes. Interestingly, T508EE had higher activity than WT kinase. While this supported the essential role of this residue in LIM kinase activity, it raised several questions. Why didn't a single glutamic acid residue activate the kinase and why did two glutamic acids give activity above WT? The work now being revised for publication in Nature Cell Biology shed some light on the problem. It seems that Thr508 was not only required for high activity, but was required for phosphorylation and activation by Pak. Both mutation of Thr508 to T508EE and Pak binding to LIM kinase and phosphorylation of this residue would result in the addition of two negative charges to the surface of the kinase domain. While Pak activated LIM kinase phosphorylation of cofilin >10 fold over non-activated LIM kinase, the T508EE mutation resulted in less than a 2 fold activation, *in vitro*. A possible explanation may be that when Pak bound to and phosphorylated LIM kinase, it bound to the highly basic region. This may function to dissipate or obscure the highly basic charges within the insert. We theorize that the exposed basic residues in the basic insert partially counteract the negative charges that are added with the T508E and T508EE mutations. This could explain why LIM kinase

is activated by Pak binding and phosphorylation to a greater extent than with mutations that mimic phosphothreonine. This is supported by data showing that T508EE LIM kinase, which is able to bind Pak, had increased activity *in vivo*.

The basic insert was hypothesized to be the region involved in the docking of the atypical phosphorylation site on cofilin to the kinase core of LIM kinase, but the fact that *in vitro*, mutation or deletion of this entire region had no effect on cofilin phosphorylation suggests that this theory is incorrect. Interestingly, mutation or deletion of this region completely inhibited LIM kinase from inducing actin aggregation and bundling *in vivo*. This disparity was identical for the T508 mutants. The apparent contradictory data was explained by the data in chapter 3 showing that this region was essential for LIM kinase binding to and being activated by Pak. The contradiction between *in vivo* and *in vitro* data can be explained by understanding that the LIM kinase purified from various cell lines and expression systems is unphosphorylated, unactivated, basal LIM kinase, while the LIM kinase that induces actin aggregation in a cell is LIM kinase that has been phosphorylated and activated by endogenous Pak. This hypothesis would explain why there is very little if any difference between the *in vitro* basal activity of LIM kinase mutants that can and cannot be activated by Pak and dramatic differences in the ability of these same mutants to induce actin bundling *in vivo*.

Key Research Accomplishments

- Identification of regulatory domains within LIM-kinase that regulate effects on the actin cytoskeleton
- Identification of domains within the kinase domain of LIM-kinase that are necessary for effects on the actin cytoskeleton
- Identification of LIM-kinase within the actin reorganization pathways controlled by Rac and Cdc42
- Identification of the upstream activating kinase that regulates LIM-kinase activity in vivo
- Identification of a novel, naturally occurring form of LIM-kinase that possesses a dominant negative phenotype with respect to LIM-kinase function

Reportable Outcomes

- One manuscript published, one in review
- PhD thesis work supported and obtained
- Several cell lines and virus strains expressing or capable of inducing expression of recombinant LIM-kinase
- Identification of novel LIM-kinase splice variant with dominant negative properties
- Postdoctoral fellowship position obtained based on work supported by this award

Conclusions

The roles of the Rho family of GTPases in the regulation of the actin cytoskeleton have been extensively studied, yet the mechanisms directly connecting Rho family effectors to proteins actively involved in cytoskeletal organization have not been determined. The interaction of Pak with Rac and Cdc42 has been studied and both have been shown to activate Pak *in vivo*, though the subsequent effects of this activation on the cytoskeleton were unknown. The work presented here connects the regulation of the cytoskeleton by the GTPases, Rac and Cdc42, through Pak to LIM kinase and cofilin. Rac and Cdc42, when activated by bound GTP, binds to the p21 binding domain, resulting in a conformational change which removes the amino terminal regulatory domain from binding to and inhibiting the kinase domain (Figure 1). The activated Pak then binds to and activates (through phosphorylation of Thr508) LIM kinase which also has an amino terminal regulatory domain. The activation of LIM kinase by Pak phosphorylation is dependent more on phosphorylation at Thr508 than on removal of the amino terminal inhibitory domains. This >10 fold activation of LIM kinase activity towards its *in vivo* substrate cofilin, results in a decrease in active cofilin and a subsequent stabilization of actin polymers within a cell. We showed that Pak activation of LIM kinase connects Rac and Cdc42 to the cellular machinery that controls actin cytoskeletal reorganization. The cross talk between the Rho family of GTPases and the presence of effectors common to more than one GTPase suggests that many of the

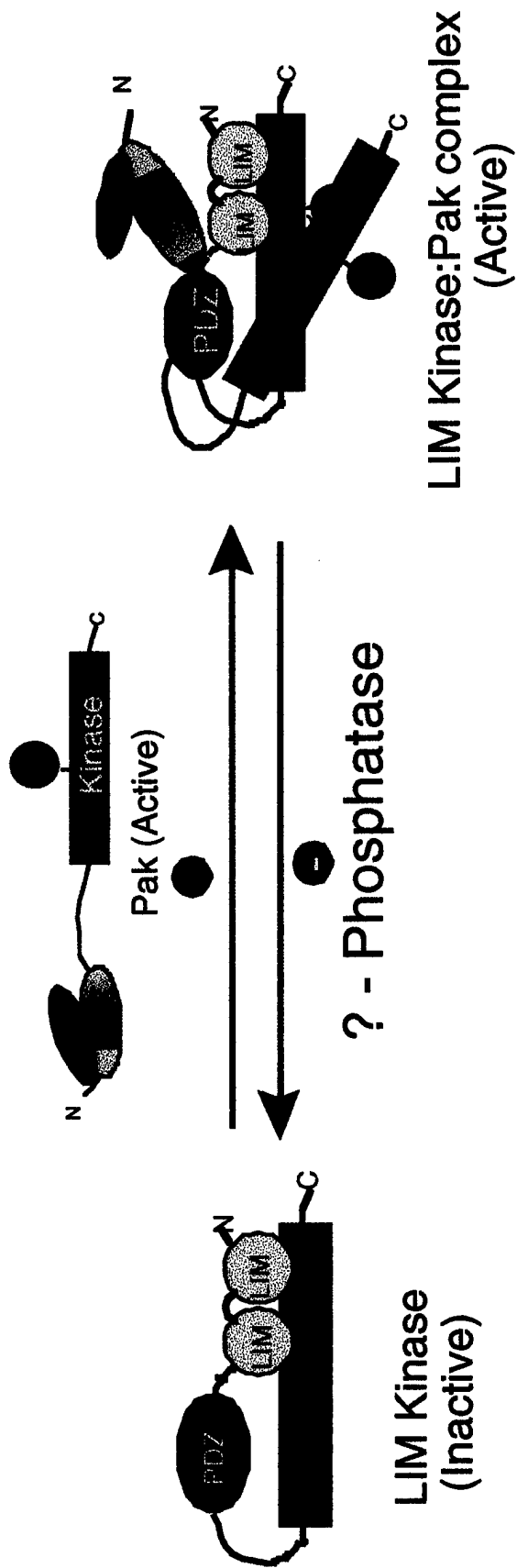


Figure 1 Pak Activation of LIM Kinase Connects Rac and Cdc42 GTPases to the Regulation of the Actin Cytoskeleton

In an unactivated state, the LIM domains bind to the kinase domain within the same polypeptide or in another LIM kinase peptide. The PDZ domain binds to an inhibitor protein and/or serves to localize LIM kinase to a different subcellular localization. Thr508 within the kinase domain does not contain a phosphate. Extracellular signals activate Rac or Cdc42, resulting in Pak activation and LIM kinase phosphorylation. This activates the kinase 10 fold, but the PDZ and LIM domains still bind to their substrates, so additional activation is possible. Additional signals or proteins may serve to release the LIM domains from binding the kinase domain and the PDZ domain from the inhibitor or subcellular anchor. LIM kinase activation induced by Pak is regulated by GEFs, GAPs, GDIs and phosphatases.

mechanisms used to alter the actin cytoskeleton are not unique to individual GTPases. The peripheral cytoskeletal reorganization seen in both Rac and Cdc42 activated cells suggests that they use common cytoskeletal control mechanisms. The ability of a dominant negative LIM kinase to interfere with this common reorganization, suggests that both of these GTPases signal through LIM kinase to induce this phenotype. While activation of LIM kinase by Pak and Rac is sufficient to induce membrane ruffling, it is not sufficient to induce filopodia formation, suggesting that other mechanisms downstream of Cdc42 are involved in filopodia formation .

Additional signals regulate the functions of all members of this pathway. The LIM and PDZ domains on LIM kinase and the SH3 binding sites on Pak are members of families of domains that have been repeatedly shown to be involved in protein regulation. The kinase cascade shown here to activate LIM kinase and inhibit cofilin activity must also be down regulated by the appropriate phosphatases. If all the cofilin were phosphorylated, there would be no more regulation of actin polymerization, the free pool of actin monomers would be consumed and a cell would sit frozen, unable to move, grow, divide, endocytose, etc.. The phosphatases regulating Pak, LIM kinase and cofilin phosphorylation are undoubtedly independent phosphatases, each regulated by there own tightly controlled signaling pathways. It would be short sighted to think that the signaling pathways involved in regulating the actin cytoskeleton or even just the polymerization of the actin cytoskeleton, have been fully characterized.

Based on the data presented here, we propose a model where Rac and Cdc42 regulate the pool of active cofilin by regulating the activity of LIM kinase through the

effector Pak. This research has provided the first evidence that directly connects the Rho family of GTPases with proteins involved in the regulation of the actin cytoskeleton.

Final Thought

The polymerization of actin plays key roles in many functions within a cell and without exquisite, careful regulation, these complex pathways could not function. The effect of LIM kinase expression and its position downstream of Rac and Cdc42 suggests that LIM kinase plays a key role in many of these processes. The prominent expression in adult neurons, the inhibition of Ras transformation and the disruption of the highly regulated cytoskeletal structures necessary for growth and division seen with LIM kinase expression, suggests that LIM kinase may function to keep cells from growing and dividing and may play key functions in diseases like cancer and AIDS. Indeed, cancer progression is just a combination of normal cellular events that progress without control and regulation. Additionally, genetic developmental disorders like Williams syndrome which physically manifests itself as a lack of differentiation and growth of a whole subset of neurons in the brain, occurs with just decreased levels of LIM kinase and suggests that tight regulation of LIM kinase expression is necessary for regulation of cell growth. LIM kinase may prove to be a potential target for treatment of these and other diseases where access to, or growth of, a specific subset of cells is essential for disease progression.

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

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LIM kinase phosphorylates and inactivates the actin binding/depolymerizing 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 is expressed early in neuroectoderm, cardiac mesoderm, and gut endoderm and later predominantly in brain (5–7). In developing human tissues LIM kinase is also found predominantly in brain where hemizygous deletion of the 7q11.23 region containing LIM kinase is implicated in the visuospatial 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). Ser³ 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 Ser/Thr-X-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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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF134379.

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structural features of cytoskeletal regulatory proteins and kinase activity specific for cofilin.

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 cofilin. 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)¹ products were amplified with Pfu polymerase. Cofilin was a kind gift from Laurent Blanchoin 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 reactions (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'-GTACTAAGCTTCATGAGGTTGACGCTACTTTGT-3' for the 5' end, and 5'-GTACTAAGCTTCAGTCGGGGACCTCAGGGTGGGCAAGG-3' for the 3' end. The PCR products were amplified with Pfu polymerase, digested with *Hind*III, and ligated into the *Hind*III 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, Willinghamshire, United Kingdom).

To determine the expression pattern of the dLIM kinase splice variant, 18 libraries were screened using RT-PCR. Four libraries were obtained: human fetal brain (a kind gift from Dimitri Krainc and R. Brent, Harvard, Boston, MA), HeLa and SK-N-MC (CLONTECH, Palo Alto, CA), human placenta and A431 (Stratagene), and 14 libraries were made from cultured cells using the Superscript cDNA Cloning Kit. Oligonucleotide primers (5' primer 5'-CCCCTGAGCTCTCCGGCT-TATACTC-3' and 3' primer 5'-CCTCCTGAGGAACGCTCTGGGT-3') were made that would amplify the region in LIM kinase that contains the deletion. PCR amplification of the region around the alternate splice site would produce a 270-nucleotide (nt) fragment, while amplification of the identical region in full-length LIM kinase would produce a 331-nt fragment. PCR reactions were then run on a 1.5% agarose gel and stained with ethidium bromide.

Site-directed Mutagenesis—All LIM kinase mutations were made by overlap extension (34, 35), except for the G177E/L178A and T508EE, which replaces Thr⁵⁰⁸ with 2 Glu residues which were made using the

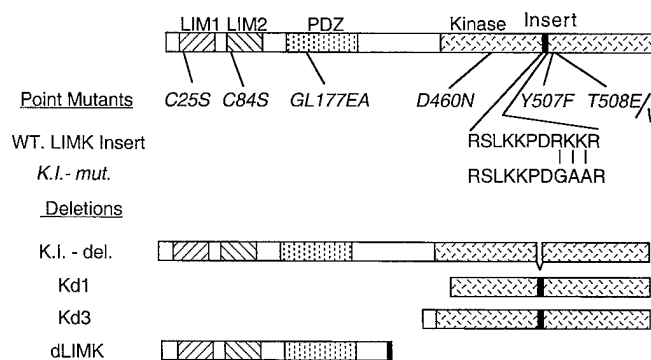


FIG. 1. Mutations in LIM kinase. The indicated mutations in LIM kinase were prepared and inserted into the pcDNA 3 expression vector as described under "Experimental Procedures." Point mutations are in *italics* with letters corresponding to the one-letter amino acid code. The *black box* in dLIM kinase represents 12 amino acids not present in wild-type LIM kinase. The *black bar* within the kinase domain represents the 11-amino acid insertion.

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'-GACTCATCGAGTCGGACCTCACCAGCGGAGGTGCTG-3' and 5'-GATCCTCGAGCCGGCGCTGGCTCACTGGGCTCCCCG-3', respectively, and the 3' primer 5'-GCGTCTAGAT-CAGTCGGGGACCTC-3'. The constructs were amplified by PCR and ligated into the *Xho*I and *Not*I 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 KETYYRRGESGLPAHPEVPD corresponding to the carboxyl-terminal 18 residues and a peptide that corresponds to residues 255–271, KEHDPHDTLGHGLGPETS, of human LIM kinase were conjugated to keyhole limpet hemocyanin using glutaraldehyde (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 the internal peptide, 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).

Immunocytochemistry—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 plate 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 benzamide, 2 μM aprotinin, 2 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml phenanthroline) 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-

¹ 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.

Sepharose and washed 4 times with lysis buffer. Immunoprecipitates for kinase assays were washed and resuspended in kinase assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 3% glycerol). An aliquot of immunoprecipitated LIM kinase was resuspended in SDS loading buffer and protein estimated by Western blotting using antibody 625 or anti-HA antibody. Protein was detected with goat anti-chicken or anti-mouse secondary antibody conjugated to horseradish peroxidase and visualized with chemiluminescence.

Protein A-Sepharose beads containing immunoprecipitated LIM kinase were resuspended in kinase reaction buffer and incubated for 150 min at 30 °C in kinase reaction buffer with 5 μ M ATP and 10 μ Ci of [γ - 32 P]ATP (4500 Ci/mmol), 2 mM MnCl₂, and 5 mM MgCl₂ with 25 μ M cofilin. Reactions were stopped by addition of SDS sample buffer and loading on a 14% SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed to X-Omat AR film (Kodak, Rochester, NY) for 1 h at room temperature with an intensifying screen. Films were scanned with a Molecular Dynamics Personal Densitometer SI and analysis was done using Image Quart Vd 1.1 (Molecular Dynamics). Phosphatase treatment of immunoprecipitated LIM kinase was carried out at 30 °C for 25 min with and without calf intestinal alkaline phosphatase (CIP) (New England Biolabs), in kinase reaction buffer. CIP-treated and mock treated LIM kinase was washed 4 times in kinase reaction buffer and kinase reactions were run as described.

Solution assays contained 200 μ M [γ - 32 P]ATP (specific activity 0.5 μ Ci/nmol) varying amounts of cofilin, 2 mM MnCl₂, 5 mM MgCl₂, 50 mM HEPES (pH 7.4), 150 mM NaCl, and various forms of LIM kinase expressed by transient transfection of HEK 293 cells. Reactions were incubated for the indicated times at 30 °C and terminated by spotting an aliquot onto Whatman 3MM filter disks that were placed in cold 10% trichloroacetic acid. Fillers were sequentially washed in cold/heated/cold 5% trichloroacetic acid, dried in 70% ethanol, and counted. Background values using untransfected HEK 293 cell lysates were subtracted and kinase activity was adjusted for LIM kinase expression quantitated by chemiluminescence imaging using Molecular Dynamics (Bio-Rad) hardware and software. SDS-polyacrylamide gel electrophoresis analysis indicated that >90% of radioactivity incorporated was into cofilin and that cofilin phosphorylation was not detected using untransfected lysates. Triplicate data points are shown for assays that were repeated.

RESULTS

Effects of LIM Kinase on the Actin Cytoskeleton—To investigate the functional consequences of mutations in LIM kinase, an *in vivo* 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 carboxyl 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 compared with neighboring untransfected cells (Fig. 2, A and B). Intermediate levels of LIM kinase resulted in loss of the cable-like stress fibers and the appearance of multiple irregular lattice-like actin filaments (Fig. 2, C and D). Higher levels of LIM kinase expression resulted in accumulation of large masses of actin near the cell periphery and diffuse cytoplasmic staining (Fig. 2, E and F). The highest levels of LIM kinase expression resulted in multiple discrete masses of actin (Fig. 2, G and H). The extent of changes in the actin cytoskeleton were scored using a 1⁺ to 4⁺ scale. The distribution of these actin cytoskeletal phenotypes in the population of cells expressing LIM kinase are shown in Fig. 4. As reported (2, 4), LIM kinase was expressed in the cytoplasm; at high levels of aggregation the enzyme colocalized with the masses of actin. With extensive disruption of the actin cytoskeleton, multiple nuclei were often observed, consistent with resultant defects in nuclear division.

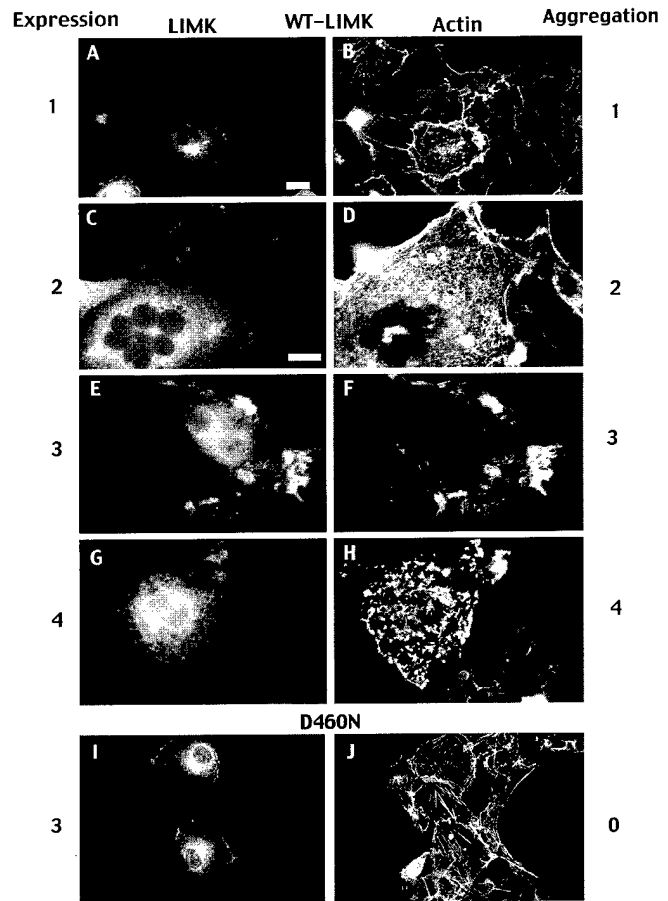


FIG. 2. LIM kinase induces an actin aggregation phenotype that is dependent on kinase activity. COS-7 cells were transfected with 1 μ g of either WT LIM kinase (A-H) or mutant kinase-inactive LIM kinase D460N (I-J). Cells were transfected, harvested, and stained with anti-LIM kinase antibodies (A, C, E, G, and I) or phalloidin (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 μ m, 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 first 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

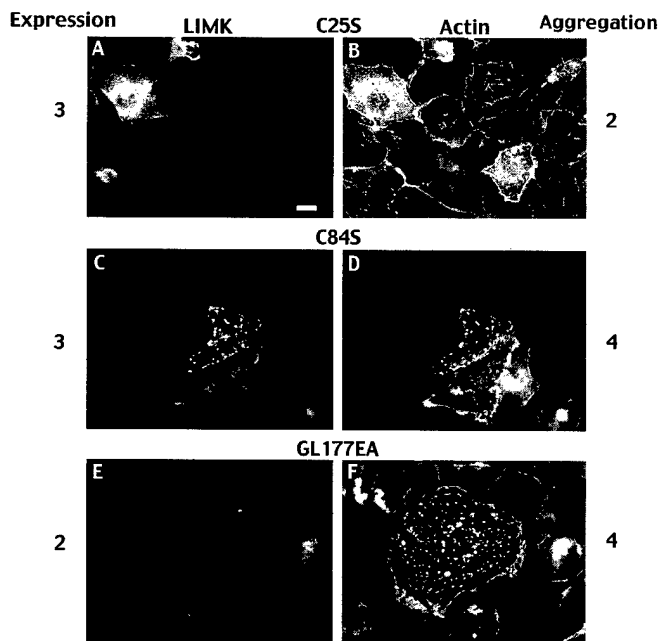


FIG. 3. The second LIM domain and the PDZ domain negatively regulate the activity of LIM kinase. COS-7 cells were transfected with 1 μ g of either LIM kinase C25S (A and B), LIM kinase C84S (C and D), or LIM kinase G177E/L178A (E and F). Cells were stained for LIM kinase (left panels) and actin (right panels), and graded on expression level (left) and actin aggregation (right).

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 holo LIM kinase 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 aggrega-

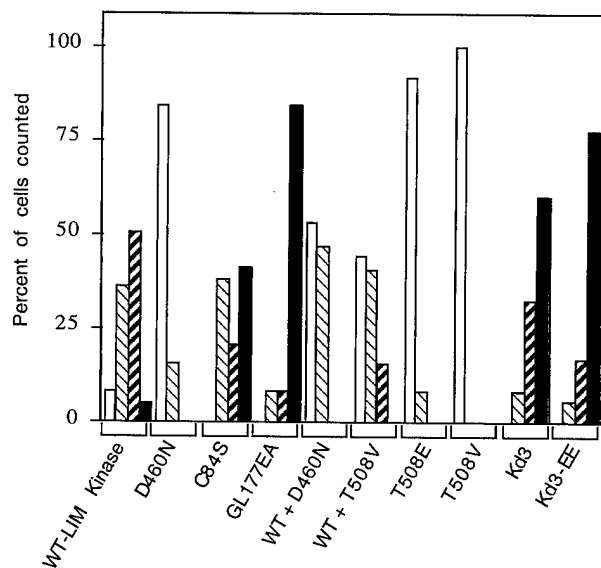


FIG. 4. Distribution of actin cytoskeletal phenotypes in cells expressing various LIM kinase mutants. COS-7 cells were transfected with the indicated LIM kinase mutants and stained for LIM kinase and actin. Transfected cells were identified and the actin phenotype scored in 100 cells without knowledge of the form of LIM kinase. □, 1⁺; ▨, 2⁺; ▩, 3⁺; ■, 4⁺, as defined in the legend to Fig. 2.

tion 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 A 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 A 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 A 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

² P. M. Guy, D. A. Kenny, and G. N. Gill, submitted for publication.

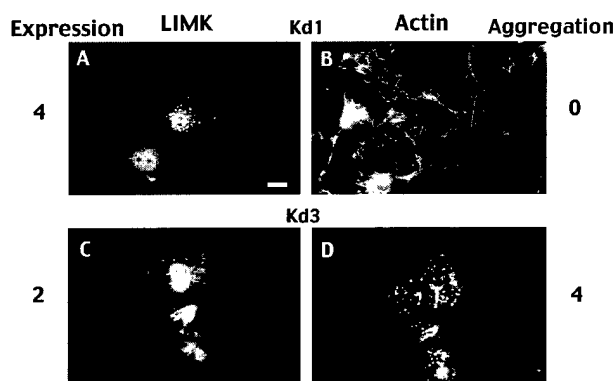


FIG. 5. The kinase domain-only construct Kd3 has high actin aggregating activity. COS-7 cells were transfected with 1 μ g of either Kd1 (A and B) or Kd3 (C and D) and stained for LIM kinase (left panels) and actin (right panels) as described under "Experimental Procedures." Expression and aggregation 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 would 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

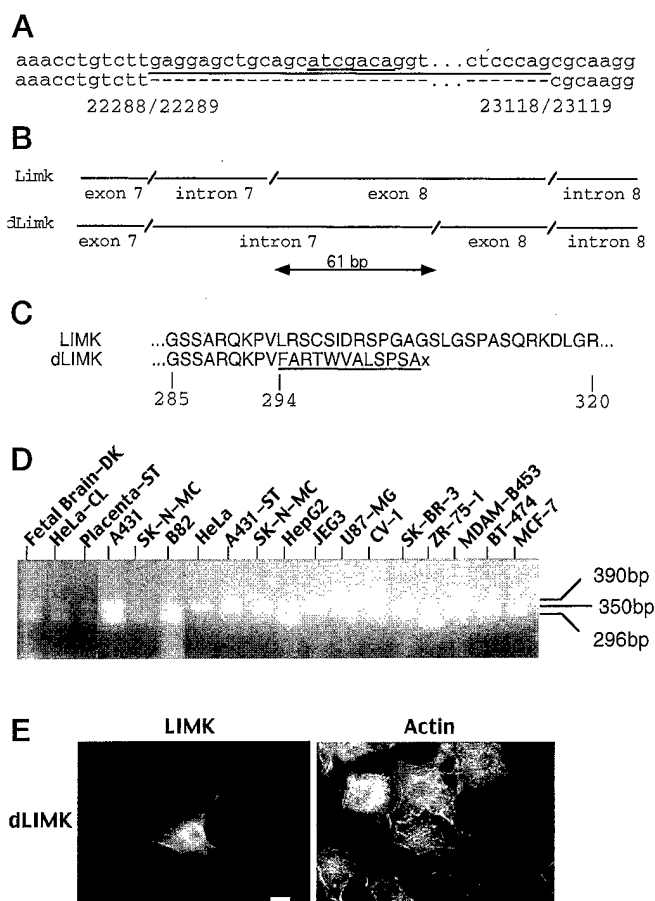


FIG. 6. A splice variant of LIM kinase results in deletion of the kinase domain. A, sequence of the splice junction of the variant dLIM kinase. The deleted sequence is underlined and the intron branch site is double underlined. 32 nt not included are indicated by dots. B, schematic of the intron and exon splicing of the full-length and dLIM kinase constructs. While holo LIM kinase joins the 3' end of exon 7 to the 5' end of exon 8, dLIM kinase joins it to the middle of exon 8. C, sequence of the carboxyl terminus of the splice variant that terminates at Val²⁹³ of LIM kinase. Twelve residues added prior to the stop codon are underlined. D, comparative expression of wild-type and variant LIM kinase in various tissues and cell lines as determined by RT-PCR. The full-length LIM kinase PCR product is 331 nt whereas the dLIM kinase PCR product is 270 nt. All cell lines were of human origin except for B82 and CV-1. Fetal brain library was made by Dimitri Krainc (DK). Libraries purchased from Stratagene (ST), CLONTECH (CL) are indicated, except SK-N-MC (lane 5) which was also from CLONTECH. E, immunofluorescence detection of the splice variant dLIM kinase expressed in COS-7 cells using the internal peptide antibody 5078 (left panel) and lack of effect on the actin cytoskeleton as determined by phalloidin staining (right panel). The sequence data are available from GenBank under accession number AF134379.

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-

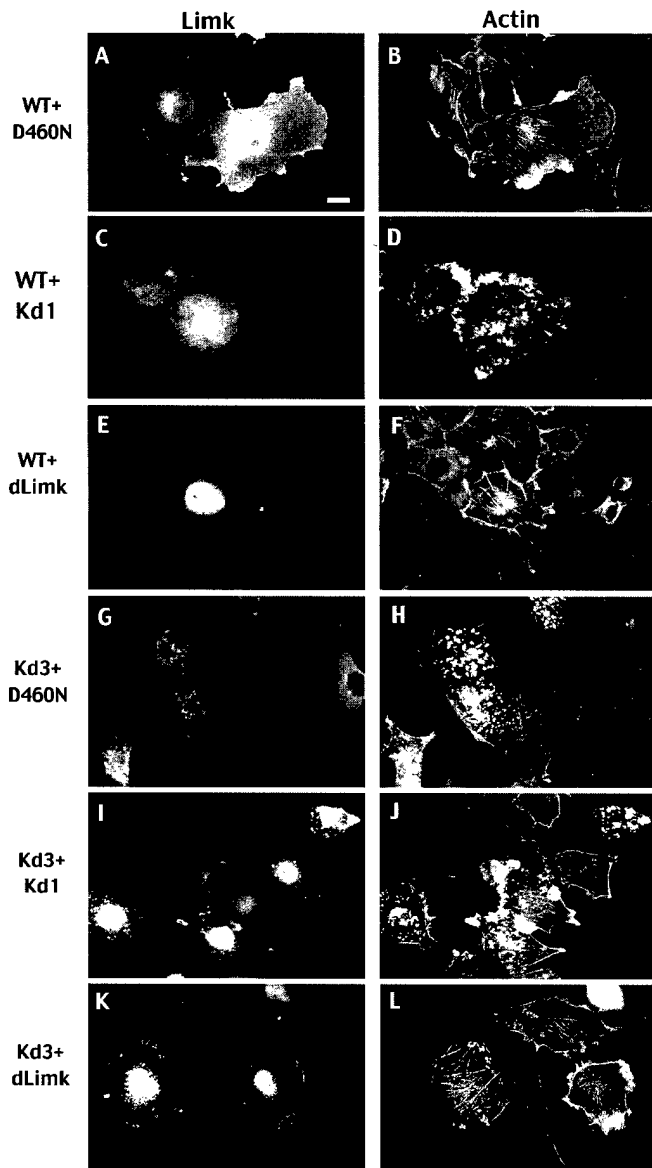


FIG. 7. The kinase inactive mutant D460N and the dLIM kinase splice variant act in a dominant interfering manner. COS-7 cells were transfected with 1 μ g of each DNA coding for LIM kinase D460N (A, B, G, and H), wild-type LIM kinase (A-F), Kd3 (G-L), Kd1 (C, D, I, and J), and dLIMK (E, F, K, and L) except for panels G and H which were transfected with 4 μ g of D460N LIM kinase. Cells were stained for LIM kinase with the 5079 antibody, to detect changes in bundling activity (left panels) and actin (right panels). In panel K dLIM kinase was detected with 5078 internal peptide antibody and Kd3 with the carboxyl-terminal 5079 antibody.

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

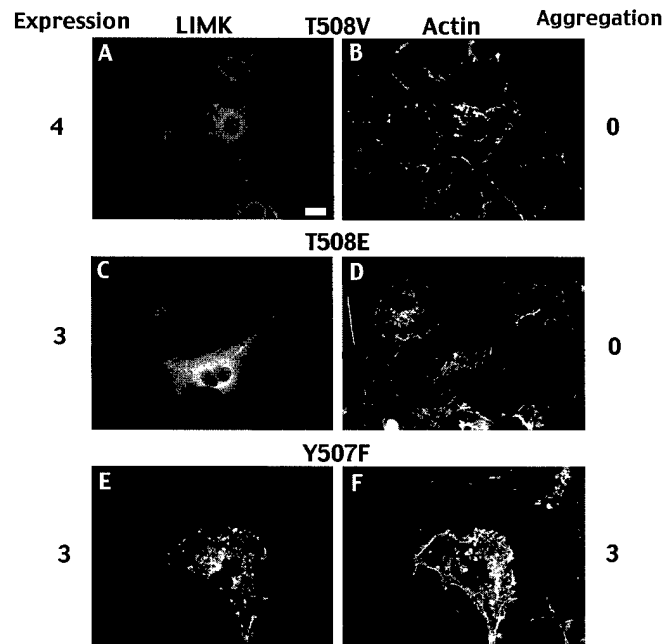


FIG. 8. Thr⁵⁰⁸ is necessary for LIM kinase effects on the actin cytoskeleton. COS-7 cells were transfected with 1 μ g of T508V LIM kinase (A and B), T508E LIM kinase (C and D), or Y507F LIM kinase (E and F). Cells were stained for LIM kinase (left panels) and actin (right panels).

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, Thr⁵⁰⁸, is adjacent to a Tyr residue, Tyr⁵⁰⁷. 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 Thr⁵⁰⁸ and Tyr⁵⁰⁷ as potential regulatory phosphorylation sites in LIM kinase, Thr⁵⁰⁸ was mutated to Val (T508V) and Glu (T508E) and Tyr⁵⁰⁷ was mutated to Phe (Y507F) 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 Thr⁵⁰⁸ 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 Thr⁵⁰⁸, 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 Thr⁵⁰⁸ is required for LIM kinase activity. In contrast changing T507F did not reduce LIM kinase activity

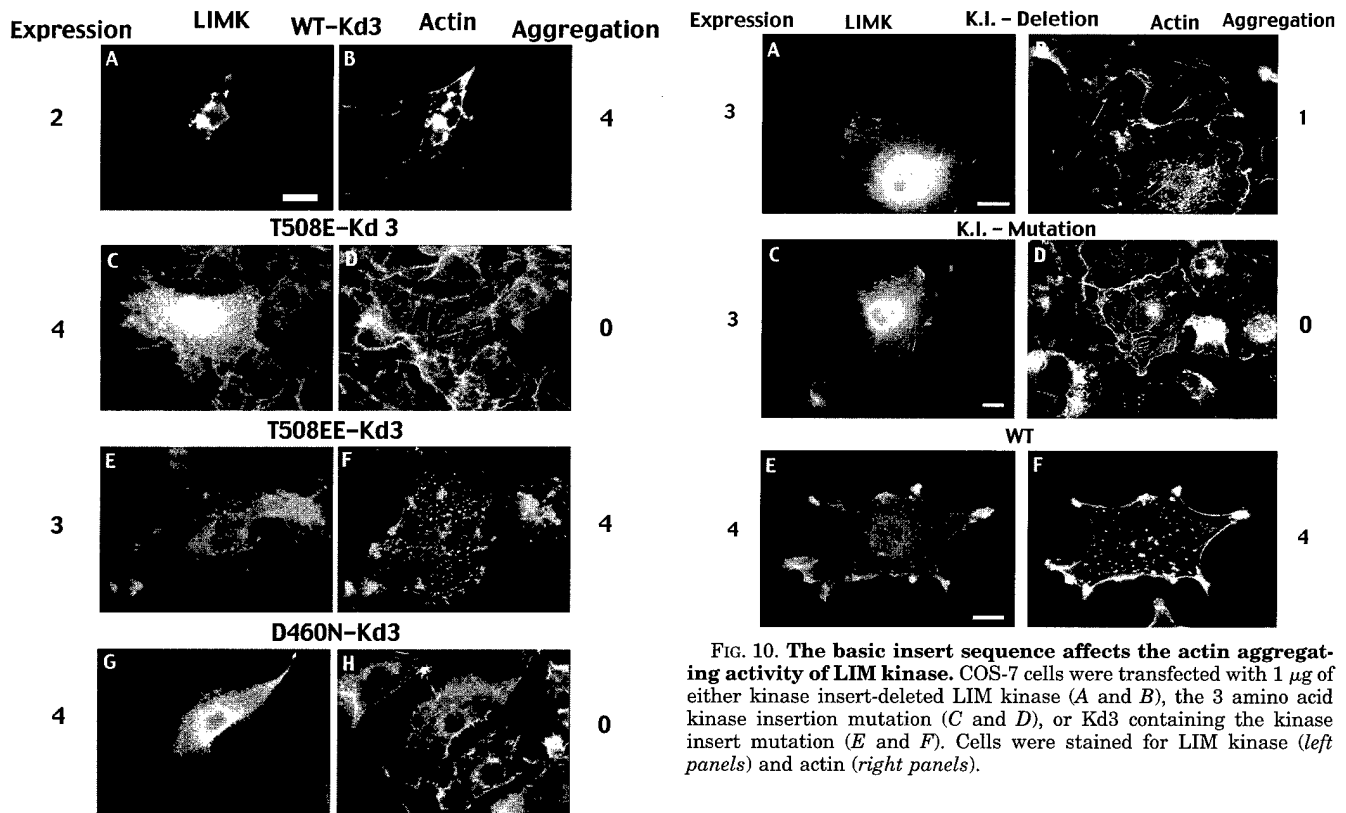


FIG. 9. Two glutamic acid residues in the activation loop activate LIM kinase. COS-7 cells were transfected with Kd3 (A and B), Kd3 containing a single Glu replacement of Thr (T508E) (C and D), Kd3 containing 2 Glu residues in this area (T508EE) (E and F) or the kinase-inactive mutation D460N in Kd3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

on the actin cytoskeleton (Fig. 8, panel E). Thus, Thr⁵⁰⁸ is likely an essential phosphorylation site in LIM kinase whereas Tyr⁵⁰⁷ is not. The failure to autophosphorylate on Thr *in vitro* (6) and the lack of kinase activity of bacterially expressed Kd3 (data not shown) suggest Thr⁵⁰⁸ is phosphorylated *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 Thr⁵⁰⁸ 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 ⁵⁰²Arg-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⁺ 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 *in vitro*. As shown in Fig. 11A, both wild-type and Kd3 LIM kinase catalyzed phosphorylation of cofilin *in vitro*. LIM kinase catalyzed phosphorylation of myelin basic protein to a

FIG. 10. The basic insert sequence affects the actin aggregating activity of LIM kinase. COS-7 cells were transfected with 1 μ g 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).

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 Thr⁵⁰⁸ to either Val or Glu markedly reduced kinase activity *in vitro* in agreement with loss of the effects of these enzymes on the actin cytoskeleton *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 *in vitro* thus paralleled effects *in vivo* indicating that Thr⁵⁰⁸ 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 *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 *in vitro* kinase assays reflect the basal activity of LIM kinase. In the absence of the kinase insert LIM kinase cannot be activated *in vivo*, resulting in the observed marked reduction in actin aggregation compared with WT LIM kinase. The insert in the activation loop along with Thr⁵⁰⁸ is thus necessary for full *in vivo* activity. The K_m of LIM kinase for cofilin is 7.6 μ M compatible with the intracellular concentration of cofilin (Fig. 11C).

To demonstrate that the phosphorylation seen *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 *in vitro*. This demonstrates that LIM kinase specifically

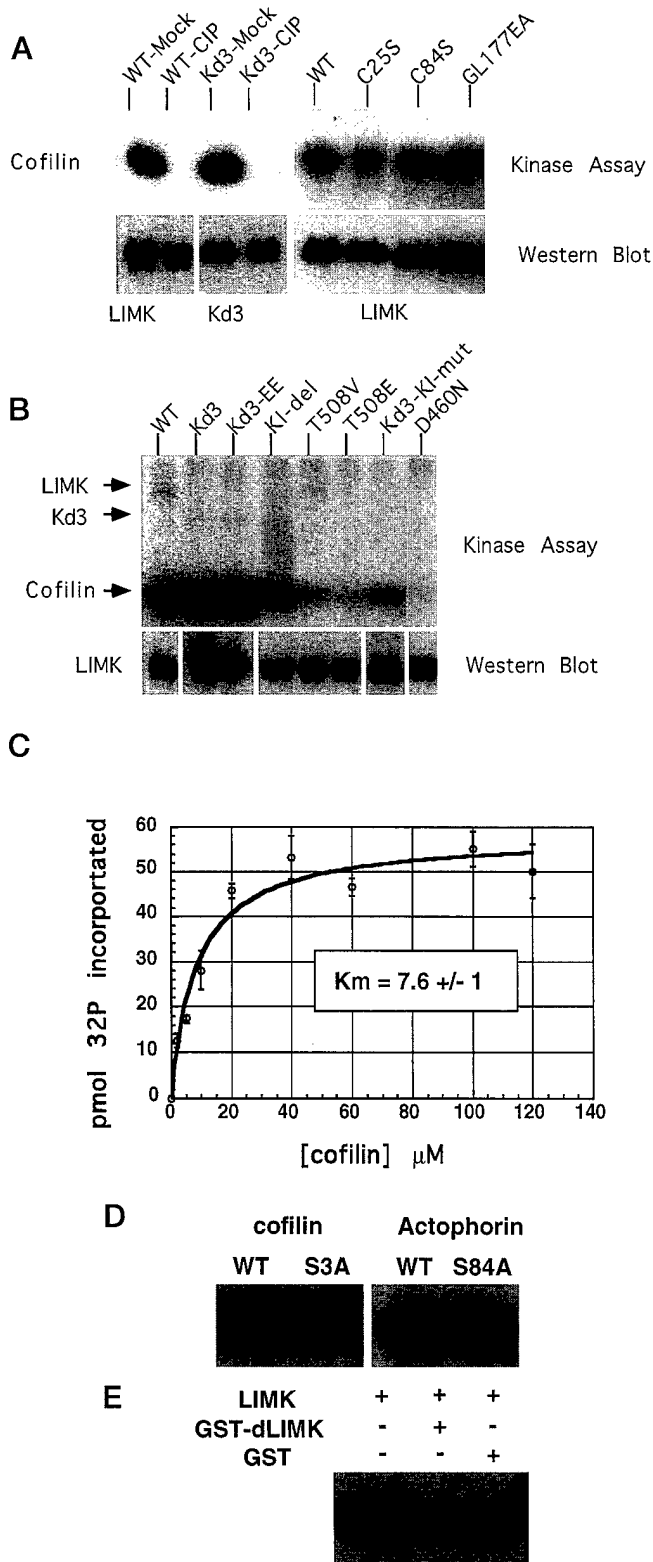


FIG. 11. *In vitro* phosphorylation of cofilin by wild-type and mutant LIM kinase. All DNAs were transfected into HEK 293 cells and lysates were used in *in vitro* kinase assays and Western blotting as described under "Experimental Procedures." **A, left:** LIM kinase inactivation by dephosphorylation. Immunoprecipitated LIM kinase and Kd3 were incubated without or with CIP, washed, and *in vitro* kinase activity assayed using cofilin and [γ - 32 P]ATP as substrates. An autoradiogram of 32 P-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

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 μ M cofilin in kinase reaction mixtures for 6 min at 30 $^{\circ}$ C and 32 P 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 [32 P]phosphate incorporated)	Relative activity ^a
WT	34.8 \pm 3.2	1.0
C25S	32.1 \pm 2.8	0.9
C84S	33.1 \pm 3.0	1.0
G177E/L178A	38.2 \pm 2.5	1.1
D460N	0.0	0.0
T508V	11.8 \pm 4.2	0.3
Y507F	33.3 \pm 3.6	1.0
Kd3	82.6 \pm 9.2	2.4
Kd3-del	81 \pm 7.5	2.3
Kd3-EE	114.9 \pm 10.8	3.3

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

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

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 K_m for cofilin. The indicated concentrations of cofilin were added to solution assays containing Kd3 and incubated for 12 min at 30 $^{\circ}$ C. Incorporation of 32 P into cofilin was quantitated as described under "Experimental Procedures." **D,** phosphorylation of Ser³ of cofilin. Lysates of 293 cells transfected with WT LIM kinase were used in kinase assays containing WT and mutant cofilin (serine 3 to alanine, S3A) and WT and mutant actophorin (serine 84 to alanine, S84A). Two μ g of substrate were used in each reaction. **E,** 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 LIM1 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 carboxyl-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 reg-

ulate 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, ⁵⁰⁴Lys-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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Activation of LIM-kinase by Pak Couples Rac/Cdc42 GTPase Signaling to Actin Cytoskeletal Dynamics

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Abstract

Extracellular signals regulate actin dynamics through small guanosine triphosphatases (GTPases) of the Rho-Rac-Cdc42 (p21) family. The p21-activated kinase (Pak1) phosphorylated LIM-kinase at threonine 508 within the activation loop and increased LIM-kinase phosphorylation of the actin regulatory protein cofilin ~10-fold in vitro. In vivo, activated Rac or Cdc42 increased association of Pak with LIM-kinase, which required structural determinants in both the NH₂-terminal regulatory and COOH-terminal catalytic domains of Pak. A catalytically-inactive LIM-kinase interfered with Rac-, Cdc42- and Pak-dependent cytoskeletal changes. A Pak-specific inhibitor, corresponding to the Pak autoinhibitory domain, blocked LIM-kinase-induced cytoskeletal changes. Activated GTPases thus can regulate actin depolymerization through Pak and LIM-kinase.

Introduction

The actin cytoskeleton is a dynamic structure where the rates of polymerization and depolymerization of actin are important for cell motility, cell division and the formation of specialized structures. **Actin polymerization drives formation and extension of protrusive structures at the leading edge of motile cells whereas myosin provides the tractive force necessary for cell movement (1,2).** The Rho family of GTPases regulate actin cytoskeletal dynamics by cycling between inactive GDP-bound and active GTP-bound states (3,4). Although cross talk among family members occurs, each is activated in response to specific environmental signals and each induces specific changes in the actin cytoskeleton: Rho induces assembly of stress fibers and focal adhesions; Rac induces peripheral actin accumulation and membrane ruffling; Cdc42 induces filopodia; both Rac and Cdc42 assemble focal complexes (5).

A number of effectors of Rho GTPases have been identified but signal transduction pathways that link these to the actin cytoskeleton are not completely understood. A number of actin-associated proteins that regulate actin polymerization and depolymerization are potential downstream mediators. The Arp 2/3 complex binds with high affinity to the pointed ends of actin filaments and nucleates actin filaments to polymerize and elongate from the barbed end (6). The Wiskott-Aldrich Syndrome family of related proteins (WASP) appear to be the principal regulators of Arp 2/3-mediated nucleation of actin polymerization (7). Analysis of actin polymerization in vitro indicates synergistic activation of actin polymerization by PI (4,5) P₂ and GTP- γ -S-Cdc42 via their binding to the NH₂ terminus of N-WASP to relieve inhibitory constraints on the COOH terminus of N-WASP that interacts with Arp 2/3 and actin (8). Additionally, Rac and Rho have been shown to control the severing and nucleation of new actin filaments through gelsolin whose interaction with actin is regulated by PIP₂ (9).

The cofilin/actin depolymerizing factor family of small actin binding proteins are also important regulators of the actin cycle, mediating cleavage and disassembly of actin filaments (10). They therefore serve as an important downstream control point for cytoskeleton dynamics (11). LIM-kinase catalyzes phosphorylation of an NH₂ terminal serine residue of cofilin thereby inactivating its F-actin depolymerizing activity and leading to accumulation of actin filaments and aggregates (12,13). LIM-kinase acts downstream of Rac but is not a direct target of this GTPase (12,13). A potential link between Rac and Cdc42 and LIM-kinase is the p21-activated kinase, Pak1, which, through the Rac and Cdc42 binding domain (PBD), binds to and is activated by guanosine triphosphate (GTP)-bound Rac and Cdc42 (14,15). Pak1 has been localized to regions of cytoskeletal assembly (16,17), and activated forms induce cytoskeletal rearrangements independent of Rac or Cdc42 activation (16-19).

In the present studies we tested whether Pak1 could activate LIM-kinase and used inhibitors of Pak1 and LIM-kinase *in vivo* to investigate the connections identified *in vitro*. The results define a signal transduction pathway in which GTP bound-Rac-or Cdc42-activated Pak1 transphosphorylates and activates LIM-kinase which in turn efficiently catalyzes phosphorylation and inactivation of cofilin, resulting in decreased depolymerization of F-actin.

Results

Activation of LIM-kinase by Pak1

LIM-kinase activity was measured *in vitro* as ³²P incorporation into Ser³ of cofilin (20). Addition of a constitutively active GST-Pak1 (Pak*) (15) enhanced the rate of LIM-kinase-catalyzed phosphorylation of Ser³ of cofilin 7.6-fold (Fig. 1A). Neither Pak* alone, nor a catalytically-inactive mutant of LIM-kinase (D460N) in the presence of Pak*, caused

phosphorylation of cofilin. The NH₂-terminal LIM and PDZ domains of LIM-kinase suppress the COOH-terminal kinase domain and inhibit LIM-kinase-induced cytoskeletal changes (20). The kinase domain of LIM-kinase alone (Kd, residues 302 to 647) had ~2.5-fold greater activity than full-length (WT) LIM-kinase, and also was activated 7.6 fold by Pak* (Fig 1A). The stimulatory effects of Pak* on LIM-kinase are thus directly due to effects on the kinase domain.

To investigate the mechanism of activation of LIM kinase by Pak*, the effects on cofilin phosphorylation of several mutations in the kinase domain of LIM-kinase were tested. LIM-kinase contains a highly basic 11 amino acid insertion in the activation loop (Arg⁴⁹⁵ to Arg⁵⁰⁶) (21,22) preceding a threonine (Thr⁵⁰⁸) that is analogous to a regulatory phosphorylation site in other protein kinases (23,24). Mutation of Thr⁵⁰⁸ to valine (T508V) reduced LIM-kinase activity whereas replacement with two glutamic acid residues (T508EE) resulted in an active form of LIM-kinase; neither T508V nor T508EE were activated by Pak* (Fig. 1B). Both deletion and mutation of the 11-amino acid basic insert completely disrupted Pak* activation of LIM-kinase (Fig. 1B). In contrast, mutation of Tyr⁵⁰⁷ did not affect LIM-kinase activation by Pak* (data not shown). These results suggest that Pak* activates LIM-kinase through phosphorylation of Thr⁵⁰⁸, with the basic insert providing the substrate determinants required by Pak (25).

Pak* phosphorylated the purified kinase domain (Kd) of LIM-kinase coincident with activation (Fig. 1C). MALDI-mass spectroscopy identified an 80-Da shift (2098 Da to 2178 Da) in the tryptic peptide corresponding to the activation loop of LIM-kinase (⁵⁰⁷YT*VVGNPYWMAPGMINGR) after phosphorylation of purified Kd by Pak. This confirmed that Thr⁵⁰⁸ was the site phosphorylated by Pak*. Additionally, Pak* phosphorylated a catalytically-inactive form of Kd (D460N Kd), but not the Kd-EE mutant (data not shown). These

results coupled with the low activity of the T508V mutant and loss of LIM-kinase activity upon phosphatase treatment (20) indicate that phosphorylation of Thr⁵⁰⁸ is required for maximal LIM-kinase activity. Neither protein kinase C β II, protein kinase A nor phosphoinositide-dependent protein kinase 1 were able to phosphorylate or activate LIM-kinase.

Binding of activated Rac or Cdc42 to the NH₂-terminal p21-binding domain (PBD) in Pak induces a conformational change that relieves inhibitory constraints on the COOH-terminal kinase domain (14,15,26). The active Pak kinase domain [amino acids 232 to 504, Pak-KD(Kin⁺)] enhanced LIM-kinase activity ~2-fold (Fig 2A, left panel). Neither the catalytically-inactive kinase domain [Pak-KD(Kin⁻)] (Fig 2A, left panel) nor the full-length catalytically-inactive GST-Pak (K299R) (Fig 2A, right panel) activated LIM-kinase. Although an NH₂-terminal PBD-containing fragment of Pak (Pak amino acids 67-150) alone had no effect, the full-length Pak* activated LIM-kinase more effectively than the kinase domain only construct Pak-KD(Kin⁺) (Fig2A, left panel), suggesting that the presence of an intact NH₂ terminus of Pak enhances activation.

Histidine-tagged full-length Pak1 (WT-Pak) is not constitutively active and remains GTPase-dependent for full activity (27). WT-Pak alone did not activate LIM-kinase (Fig 2B). Addition of inactive GDP-bound Cdc42 to WT-Pak activated LIM-kinase ~2-fold whereas active GTP- γ -S-bound Cdc42, activated LIM-kinase ~11-fold, increasing LIM-kinase activity from 5.5 +/- 0.4 to 64 +/- 0.9 mol phosphate incorporated per mol of enzyme per minute. This is comparable to activities reported for other purified serine/threonine kinases (28). GTP- γ -S-bound Rac similarly induced Pak activation of LIM-kinase activity (data not shown). Thus Rac and Cdc42 GTPases may be connected with the actin cytoskeleton through the effectors Pak and LIM-kinase.

Rac/Cdc42-dependent interaction of Pak1 with LIM-kinase

Coimmunoprecipitation was used to investigate *in vivo* interactions between LIM-kinase and Pak. Pak was immunoprecipitated from A431 cells and immunoprecipitates were probed with a chicken antibody directed to an internal peptide in LIM kinase. As shown in Fig 3A the two proteins are associated *in vivo*. To investigate factors that regulate the association between Pak and LIM-kinase, we utilized co-transfections in 293 cells. Constitutively activated Q61L-Rac enhanced complex formation between LIM-kinase and Pak compared to inactive T17N-Rac (Fig 3B lanes 1 and 2). The partially activated H83,86L Pak1 mutant that is defective in Rac and Cdc42 binding (18) also bound LIM-kinase (Fig 3B lane 3). Complex formation was additionally observed between Pak1 and the kinase domain of LIM kinase, consistent with the ability of Pak1 to catalyze phosphorylation and induce activation of Kd.

The sites of interaction between LIM-kinase and Pak1 were mapped by binding of ³⁵S-labeled LIM-kinase to Pak1 fragments expressed as GST fusion proteins (Fig. 3C). LIM-kinase bound specifically to both the Pak1 NH₂-terminal regulatory and the COOH-terminal catalytic domains. The LIM-kinase binding region in the Pak1 NH₂ terminus localized to the region between amino acids 67 and 150, which contains the p21 binding domain (aa. 67 to 86) and the autoinhibitory regulatory domain (aa. 83 to 149), suggesting that activated Rac or Cdc42 binding would regulate LIM-kinase binding to Pak1. Addition of activated Cdc42 to WT-Pak1 or to the Pak1 PBD (aa. 67-150) enhanced binding to LIM-kinase, but did not effect the interaction with the non-p21 binding mutant H83,86L Pak1, nor with the COOH-terminal kinase domain of Pak1 (Fig. 3C). In a converse experiment Cdc42-GTP- γ -S enhanced binding of soluble WT-Pak to GST-LIM-kinase (data not shown). Thus LIM-kinase binds to a region that includes the Pak autoinhibitory domain and this interaction is increased by Rac or Cdc42 binding to the PBD.

In vivo effects on the actin cytoskeleton

To assess the role of LIM-kinase as a downstream mediator of Rac and Cdc42 in actin cytoskeletal reorganization, BHK cells were co-transfected with WT- or catalytically-inactive D460N-LIM-kinase and constitutively active Q61L-Rac or Q61L-Cdc42. While both Rac and Cdc42 induced a similar peripheral reorganization and thickening of tangential actin fibers, Rac induced prominent membrane ruffles while Cdc42 induced microspikes (Fig 4). WT-LIM-kinase expression increased the number and size of peripheral actin fibers. D460N-LIM-kinase expression disrupted Rac- and Cdc42-induced peripheral actin reorganization and Rac-induced membrane ruffling, but did not affect Cdc42-induced microspike formation. Quantitatively peripheral reorganization and thickening of actin fibers was observed in ~50% of the cells expressing Q61L-Cdc42, in ~80% of cells expressing Q61L-Cdc42 plus WT LIM-kinase but in only 5% of cells expressing Q61L-Cdc42 plus D460N-LIM-kinase. D460N-LIM-kinase did not significantly reduce Cdc42-induced microspikes in these cells. Like D460N, catalytically inactive T508V-LIM-kinase also inhibited Rac-induced actin reorganization (data not shown). Together, these data suggest that LIM-kinase is downstream of Rac-induced cytoskeletal signaling, but only mediates a subset of Cdc42-induced cytoskeletal changes.

To determine whether the LIM-kinase-induced cytoskeletal changes depended on Pak1 activity BHK cells were transfected with LIM-kinase and the NH₂-terminal autoregulatory domain of Pak1 (83-149 Pak) which inhibits Pak activity in vivo (26,29). Although control and 83-149 Pak-expressing BHK cells had a very organized actin cytoskeleton composed of parallel actin fibers, cells expressing LIM-kinase had an exaggerated membrane ruffling phenotype and accumulated large aggregates of actin (Fig 5, panel A versus C). These changes induced by LIM-kinase presumably reflect severe inhibition of depolymerization of F-actin due to inactivation of cofilin (12,13,20) Co-expression of 83-149 Pak with LIM-kinase resulted in substantial inhibition

of LIM-kinase-induced cytoskeletal changes (Fig 5, panel D). Changing Leu 107 to Phe, which inactivates the autoinhibitory domain of Pak (26), abolished the ability of the 83-149 Pak autoinhibitor to block LIM-kinase-induced cytoskeletal changes (Fig 5, panel E versus D). The distribution of actin cytoskeletal phenotypic changes was scored to provide an assessment of the effects of the 83-149 Pak inhibitor on LIM-kinase-induced changes (20). When WT LIM-kinase and the inactive L107F Pak inhibitor were co-expressed, ~80% of the cells exhibited moderate to severe changes in the actin cytoskeleton consisting of loss of stress fibers and increasing accumulation of aggregates of actin (2+ to 4+ as described in ref. 20). In contrast, when WT LIM-kinase and the 83-149 Pak inhibitor were co-expressed, ~80% of the cells exhibited no or minimal actin cytoskeletal changes (0 to 1+ as described in ref 20) These data indicate that LIM-kinase activity is dependent on Pak activity in vivo. Previous studies showed that changing Thr 508 to Val or removal of the basic insert in the kinase domain of LIM-kinase abrogated activity in vivo (20). As shown in Fig 1B, deletion of the basic insert abolished activation by Pak1 but did not abolish basal kinase activity in vitro. Together these results indicate a strict requirement for phosphorylation of Thr⁵⁰⁸ for in vivo activity of LIM-kinase. Blocking LIM-kinase-induced cytoskeletal changes with the intact but not the mutant Pak autoinhibitor supports the conclusion that Pak activity is essential for LIM-kinase activity in vivo.

Inhibition of Rac-and Cdc42-induced cytoskeletal changes by catalytically-inactive D460N-LIM-kinase, coupled with inhibition of LIM-kinase-induced cytoskeletal changes by the Pak inhibitor, support the sequence of information transfer being from Rac to Pak to LIM-kinase. If this is correct, then dominant negative LIM-kinase should interfere with Pak-induced cytoskeletal changes. The H83,86L Pak1 mutant is constitutively active and induces cytoskeletal changes, including dorsal membrane ruffles, formation of lamellipodia, small actin aggregates, cell

polarization and focal complex formation (17-19). In BHK cells the prominent phenotypes induced by the H83,86L Pak mutant included large dorsal ruffles, thickening of actin cables and aggregation (Fig 6 A and B). Similar formation of dorsal ruffles that contain Pak1 has been shown to occur with PDGF and insulin treatment (17). Addition of WT LIM-kinase increased actin aggregation in cells expressing H83,86L Pak (Fig 6C). However, co-expression of dominant negative LIM-kinase blocked both formation of the large dorsal ruffles and the actin aggregates induced by H83,86L Pak (Fig 6D). Because the level of expression of transfected proteins varies within the population of cells, formation of dorsal ruffles seen with H83,86L Pak and the actin aggregation characteristic of LIM-kinase were scored in expressor cells to provide an estimate of intrinsic activity (12,20). As shown in Fig 6G, the dominant negative D460N LIM-kinase abolished H83, 86L Pak-induced dorsal ruffles and decreased by 50% the number of cells exhibiting actin aggregates. Since previous studies indicated that dominant negative LIM-kinase blocked the actin cytoskeletal effects induced by WT LIM-kinase (12,13,20), these results indicate that LIM-kinase mediates at least some of the cytoskeletal effects of Pak.

Upon co-expression of Pak1 and LIM-kinase both proteins localized with actin in the resultant exaggerated membrane ruffles (Fig 7). The two proteins also colocalized with actin aggregates (data not shown). Co-expression of LIM-kinase with green fluorescent protein did not result in colocalization at membrane ruffles or actin aggregates (data not shown), suggesting that the *in vivo* association of LIM-kinase and Pak is specific. These findings, together with the protein interaction data shown in Fig 3, suggest that a Pak1•LIM-kinase complex is localized to the area where actin cytoskeleton rearrangements occur.

Discussion

The results presented here indicate that the cofilin-phosphorylating protein LIM-kinase is activated by the Rac- and Cdc42-dependent protein kinase Pak1. Maximal activation by Pak

requires its intrinsic kinase activity and regions in the NH₂ and COOH termini, possibly representing one constitutive and one regulated binding site. Activation of LIM-kinase depends upon phosphorylation of Thr⁵⁰⁸ in the activation loop of the kinase domain of LIM kinase by Pak. Mutational inactivation of the second LIM domain or of the PDZ domain or deletion of the NH₂ terminus containing both LIM and PDZ domains increased LIM-kinase activity in vivo (20). Moreover, the NH₂ terminus inhibited activity of the kinase domain of LIM-kinase. Relief of inhibitory constraints imposed by the NH₂ terminus is thus necessary for full activation of LIM-kinase, implying that regulatory mechanisms additional to Pak1 control LIM-kinase activity.

In agreement with the finding of Arber et al. (12) and Yang et al (13), LIM-kinase appears to mediate actin cytoskeletal responses to Rac. **The time course of insulin-induced activation of LIM-kinase is reported to parallel that of insulin-induced membrane ruffling, a process dependent on Rac (13). The time course of insulin-induced activation of Pak is similar (30). These data are consistent with ligand-dependent regulation of cytoskeleton dynamics via a Rac/Pak/LIM-kinase pathway but, because responses are localized within the cell, development of techniques to assess local changes will be necessary to precisely order physiological responses.** Moreover, while both GTP-bound Rac and Cdc42 activate Pak, catalytically-inactive LIM-kinase blocked only a part of the morphological response to Cdc42. Cdc42-induced microspikes persisted, consistent with distinct signalling pathways mediating the specific morphological responses to each of the Rho family GTPases (5). The present findings confirm those of Arber et al (12) but additionally indicate that a subset of the cytoskeletal responses to Cdc42 are mediated by LIM-kinase. Cdc42 effects on filipodia formation may be mediated by distinct pathways including N-WASP effects on nucleation of actin polymerization (31).

Pak1 has been shown to have dramatic effects on cytoskeletal dynamics and Pak function is required for both fibroblast (32) and endothelial cell motility (33), as well as formation of neurites (19). **There is evidence that Pak can regulate the actin-myosin cytoskeleton** both through NH₂-terminal-interacting proteins and by phosphorylating specific substrates. One of our laboratories has recently shown that Pak modulates cell contractility through phosphorylation and inhibition of myosin light chain kinase (34). **Previous work (18) showed that upon transient overexpression in fibroblasts, H83,86L Pak1 induced cell polarization accompanied by peripheral and dorsal membrane ruffling. When Pak1 catalytic function was inactivated, cells failed to polarize and peripheral, but never dorsal, ruffling was seen. This led to the conclusion that Pak could induce ruffling in the absence of kinase activity. More recent work by Sell et al. (35) using cell lines in which Pak1 expression could be carefully controlled, established that H83,86L Pak1 induced polarized ruffles associated with directional cell movement. In contrast, the catalytically-inactive version induced random ruffling and the cells were unable to undergo directed migration. Interestingly, in these same studies, low level expression of catalytically activated T423E Pak1 also induced a polarized ruffling phenotype. The present results using BHK cells support these findings. We observed that while expression of H83,86L Pak1 induced extensive dorsal ruffling and actin thickening, this phenotype was almost totally absent with the kinase-inactive version of the H83,86L Pak1 mutant. Instead, this mutant caused the formation of membrane extensions that were insensitive to the expression of a dominant negative form of LIM kinase.**

Our current view is that Pak can induce ruffling by at least two mechanisms: a preferred, physiologically relevant pathway that requires the kinase function and which is important for directed cell motility, and a second mechanism that is seen with the catalytically-inactive Pak. The present data establish that LIM kinase is an important

mediator of the preferred pathway. LIM kinase acts to promote and stabilize actin structures formed in response to Pak1 or other cytoskeletal effectors of Rac and Cdc42.

The present data thus define a signal transduction pathway through which GTP-bound Rac (and Cdc42) activate Pak to form a complex with and phosphorylate LIM-kinase. Activated LIM-kinase catalyzes phosphorylation and consequent inactivation of cofilin. Inhibition of actin depolymerization represents an essential control point in the overall cycle of actin polymerization – depolymerization resulting in cytoskeletal changes (11). One important pathway for transmission of extracellular signals to the actin cytoskeleton is via the Rac/Cdc42-Pak-LIM-kinase-cofilin cascade. Signaling via the WASP family of proteins that affect primarily actin polymerization (7,8) must be coordinated with effects on actin depolymerization to provide the high degree of resolution of cell responses observed in response to extracellular signals. For example, responses to Cdc42 would likely coordinately induce actin cytoskeletal changes via stimulating nucleation of actin polymerization through N-WASP and Arp2/3 and blocking depolymerization through Pak and LIM-kinase mediated inactivation of cofilin.

Methods

Preparation of LIM kinase and Pak Proteins

Cell lysates of transiently transfected HEK 293 cells, expressing either wild type (WT), catalytically-inactive dominant negative (D460N), phosphorylation site mutant (T508V), catalytically-active phosphorylation site mutant (-EE), or basic insert deletion mutant (-del) LIM kinase-1 (20) were prepared 60-72 hrs. post transfection, lysed in PBS with 0.25% Triton X-100, mixed with glycerol to 25%, and frozen at -80°C . The purified kinase domain of LIM-kinase (Kd) was prepared by cloning the kinase domain (aa. 302 to 647) of human LIM-kinase-1 into the baculovirus expression vector pAcGHLT (Pharmingen). The detergent soluble supernatant from infected SF21 cells was bound to glutathione agarose beads and digested with thrombin for 1.5 hours as per manufacturers protocols. Protein was combined with glycerol to 25%, and stored at -80°C . All Pak1 constructs were expressed and purified as described previously (15,26).

Kinase Assays

In vitro kinase reactions were run as described (20) using $200\ \mu\text{M}$ [$\gamma^{32}\text{P}$] ATP, and ^{32}P incorporation into TCA insoluble protein was measured. Kinase activity was adjusted based on protein expression level, quantitated by chemiluminescence imaging using Molecular Dynamics (BioRad) hardware and software. Phosphorylated cofilin accounted for more than 95% of the incorporated radioactivity. All assays were done in triplicate and repeated at least six times with similar results. Kinase assays for Fig. 1A and B were done using detergent soluble (0.25% Triton X-100) HEK 293 cell lysates 72 hrs after transfection. All other kinase assays were done with the purified kinase domain (Kd) of LIM-kinase. Purified Kd was

combined with stoichiometrically equivalent amounts of WT-Pak and either Cdc42 or Rac that had been loaded and activated with the non-hydrolyzable GTP- γ -S as described (15). A mutant cofilin, with the Ser³ residue replaced by an alanine, was not phosphorylated by LIM-kinase (20). Kinase reactions for SDS-PAGE analysis were stopped by addition of Laemmli sample buffer and samples visualized by autoradiography after SDS-PAGE on 13% gels.

MALDI-Mass Spectrophotometry

Mass spectrophotometry analysis of phosphorylated LIM-kinase was adapted from (36). GST-Kd from baculovirus was phosphorylated in kinase reaction buffer with a sub-stoichiometric amount of Pak* and digested with 1.5 μ g/ml of modified trypsin (Promega). The solubilized, digested proteolytic fragments were loaded on a MALDI mass spectrophotometer and analyzed for 80 Da shifts in known proteolytic fragments.

Co-Immunoprecipitation of Pak and LIM kinase

LIM-kinase, myc-Pak and myc-Rac-transfected HEK 293 cells were prepared as described for kinase reactions. The detergent soluble fraction was incubated with rabbit anti-LIM-kinase antibody 5079 (20) and Protein A-agarose for 1.5 hrs., washed, separated by SDS-PAGE and immunoblotted with mouse anti-myc monoclonal antibody 9E10 to detect Pak. The blot was stripped and LIM-kinase in immunoprecipitates was detected with the chicken anti-LIM kinase antibody 625 (20). Expressed myc-Pak and myc-Rac were identified by immunoblotting soluble lysates, and levels of protein expression were determined to be approximately equal under each condition.

³⁵S Labeling and GST-Pull Downs of LIM Kinase

LIM-kinase was labeled with ^{35}S using the Promega TNT T7 Quick Coupled Transcription/Translation system in the presence of 1 mM ZnCl_2 and [^{35}S] methionine. GST-Pak1 fusion proteins were prepared as described (15,26). For interaction assays, ^{35}S -LIM-kinase was incubated with equal amounts of various forms of GST-Pak1 bound to glutathione beads for 30 min. on ice in a 250 μL volume of 10 mM Pipes pH 7.3, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2 and then extensively washed. Where indicated, Cdc42 preloaded with GTP- γ -S was included. Bound proteins were visualized by autoradiography after SDS-PAGE on 13% gels.

Immunocytochemistry

For BHK immunofluorescence, cells were plated, grown and transfected on glass coverslips. For Cos-7 immunofluorescence, cells were plated and transfected as described (20). Pak, Rac and Cdc42 expression was detected using an anti-myc monoclonal antibody (9E10) (1:1000), while LIM-kinase was detected using the LIM-kinase specific antibody 5079 (1:1,250). Actin was detected using an Oregon Green (fluorescein) tagged phalloidin(1:200), myc-tagged Pak, Rac and Cdc42 with AMCA-S conjugated anti-mouse IgG (1:200) and LIM-kinase with Texas-Red (rhodamine) conjugated anti-rabbit IgG (1:200) (Molecular Probes, Eugene, OR) respectively. Cells were equilibrated and mounted with Pro-Long mounting media. Micrographs were taken with a Zeiss Axiophot microscope with an attached Hamamatsu color chilled CCD camera using 40x and 60x objectives.

Pak and LIM-kinase constructs expressed in BHK and HEK 293 cells were in the expression vector pcDNA-3 (Invitrogen). LIM-kinase expression vectors were transfected into BHK cells using Superfect (Qiagen, Santa Clarita, CA or Gene PORTER (GTS, La Jolla,

CA) 30 hr prior to fixation. All Pak1, Rac and Cdc42 myc-tagged constructs expressed in BHK cells were transfected using the Semliki Forest Virus (SFV) Gene Expression System (Life Technologies, Gaithersburg, MD). Virus was prepared and activated per manufacturer's instruction, and BHK-21 cells were infected in serum free media. Cells were allowed to express protein for 8-18 hours prior to experiments.

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Figure legends:

Fig. 1. **Activation of LIM-kinase by Pak in vitro.** (A) Increased LIM-kinase catalyzed phosphorylation of cofilin in the presence of Pak. LIM-kinase activity was measured without or with recombinant, constitutively active GST-Pak1 (Pak*). Equal amounts of wild type (WT), catalytically-inactive (D460N) or kinase domain only (Kd) LIM-kinase were expressed in HEK 293 cells. Cell lysates were added to reaction mixtures containing 20 μ M cofilin and [γ ³²P]-ATP and ³²P incorporation into cofilin was quantitated. (B) Requirement of the activation loop threonine and the basic insert of LIM-kinase for activation by Pak. Kinase assays were done with equal amounts of Kd or mutant Kd in which the basic insert was deleted (Kd-del) or Thr⁵⁰⁸ was changed to a valine (T508V) or to two glutamic acid residues (-EE). Activity was determined without or with Pak*. (C) Phosphorylation and activation of LIM-kinase by Pak. Kd, purified from baculovirus, was subjected to kinase assays alone or with constitutively active PKC β II or Pak*. The upper panel shows cofilin phosphorylation and the lower panel shows an autoradiogram of ³²P-labeled Kd resolved by SDS-PAGE.

Fig. 2. **Requirement of Pak activation and kinase activity for regulation of LIM-kinase.** (A) Requirement for intrinsic kinase activity and an intact NH₂ terminus of Pak for activation of LIM-kinase. Left panel: Kd-catalyzed phosphorylation of cofilin was measured without or with GST-fusions containing the NH₂-terminal regulatory domain of Pak (67-150 Pak), the active kinase domain of Pak1 [Pak-KD(Kin.⁺)], the catalytically-inactive kinase domain mutant K299R Pak1 [Pak-KD(Kin.-)] or Pak*. Relative kinase activity was calculated from linear rates of cofilin phosphorylation and the basal activity of Kd was set to one. Right panel: Kinase assays were carried out using WT LIM-kinase and cofilin without or with a constitutively active Pak1 fused to

GST (Pak1 WT or Pak* (or a catalytically-inactive mutant K299R Pak1. Reaction products were separated by SDS-PAGE and detected by autoradiography. Arrows indicate cofilin, LIM-kinase and Pak1. In addition to Pak1-catalyzed phosphorylation of LIM-kinase, both LIM-kinase and Pak1 autophosphorylate. Inset at left: Coomassie blue stained gel of the WT- and K299R-GST-Pak1 proteins used in the kinase assays. (B) Maximal stimulation of LIM-kinase by Cdc42-GTP- γ -S-activated Pak. Purified Kd was subjected to kinase assays alone, or in combination with his-tagged-Pak (WT-Pak), without or with inactive GDP bound (Cdc42-GDP) or active GTP- γ -S bound-Cdc42 (Cdc42-GTP γ S), as described in Methods.

Fig. 3. Direct interaction of LIM-kinase and Pak. (A) Association of endogenous Pak with LIM-kinase. Pak was immunoprecipitated with rabbit polyclonal anti-Pak antibody and Western blots of immunoprecipitates were probed with chicken anti-LIM-kinase antibody 625 (20) in the absence or presence of the immunizing peptide (residues 255-271 of h-LIM-kinase). The blot was stripped and reprobed with anti-Pak. (B) Increased binding of Pak to LIM-kinase in cells expressing activated Rac. The indicated combinations were transfected into HEK 293 cells. The cells were lysed after 48 hours and LIM-kinase was immunoprecipitated with the 5079 antibody against LIM-kinase. The immunoprecipitates were blotted and probed for co-immunoprecipitated Myc-tagged Pak with the 9E10 antibody against the myc tag (top panel). The blot was stripped and reprobed with the chicken anti-LIM-kinase antibody 625 to compare LIM-kinase in immunoprecipitates (second panel) and expression of myc-Pak and myc-Rac was verified by probing lysates with the 9E10 antibody (lower 2 panels). The experiment was repeated 4 times with similar results. (C) Binding of LIM-kinase to regions in the NH₂ and COOH terminus of Pak1. The indicated GST-Pak1 fragments and mutants were used to bind ³⁵S

Met-labeled full-length LIM-kinase in vitro. The amino acid residues present in each fragment of human Pak1 are indicated. Also tested were a non-GTPase binding full length Pak1 (H83,86L) (18) and full length wild type Pak1 (Pak1WT). GST beads as well as beads to which GST-Nck were bound (data not shown) did not specifically bind LIM-kinase. The right panel shows use of GST fusions of H83,86L Pak1, an NH₂-terminal fragment of Pak1 (67-150) containing the p21 binding domain (PBD) and autoinhibitory domain (AI), WT-Pak1 and the COOH terminal catalytic domain (232-544) to determine the effects of GTP- γ -S Cdc42 (Cdc42 γ S)-induced activation of Pak on LIM-kinase binding in vitro. The brackets indicate the Pak1 fragments, while the + or - indicates qualitatively the relative amount of binding to LIM-kinase. **GST-WT Pak bound 5% of the input in vitro translated [³⁵S] •LIM-kinase whereas GST, GST-Nck or GST Pak 147-231 bound < 0.5% of the input counts. The experiments were repeated with internal controls 3 times with similar results.**

Fig. 4. Inhibition of Rac- and Cdc42-induced cytoskeletal changes by dominant negative LIM-kinase. BHK cells were stained for actin with fluorescently labeled phalloidin, and cells expressing Rac, Cdc42 and WT- or D460N-LIM-kinase constructs were identified by immunostaining as described in Methods. Cells expressing Rac or Cdc42 only are indicated by an arrowhead; arrows show cells expressing both the GTPase and LIM-kinase. Fifty to one hundred cells from three independent transfections were scored for each panel.

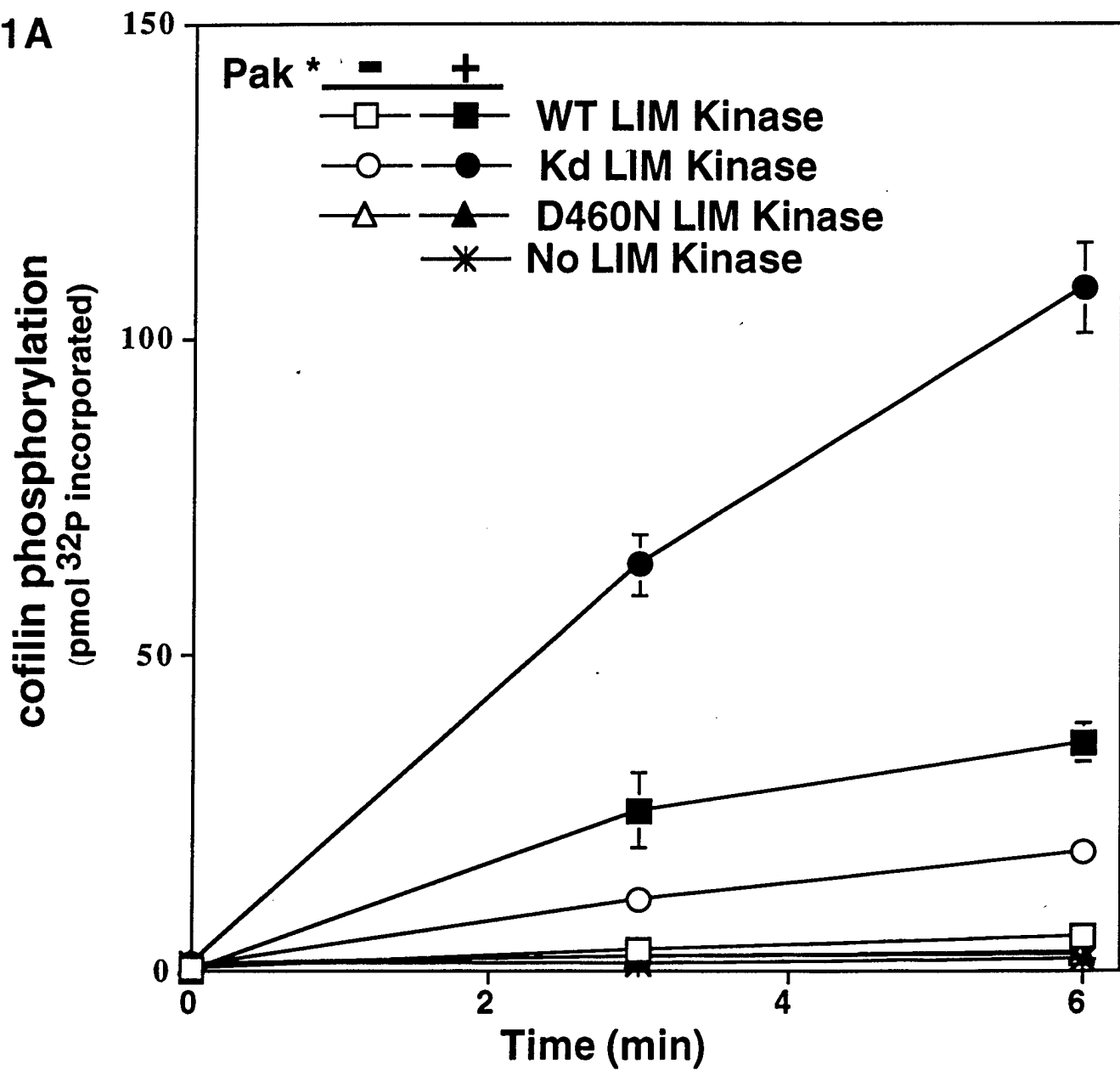
Fig 5. Inhibition of LIM-kinase-induced actin cytoskeletal changes by the autoinhibitory domain of Pak1. BHK cells were transfected with WT-LIM-kinase and either the Pak1 autoinhibitory domain (83-149 Pak) or an inactive autoinhibitory domain (83-149 [L107F]-Pak)

as described in Methods. Arrows show cells expressing the indicated protein(s). Fifty to one hundred transfected cells were scored for actin cytoskeletal changes as described (20).

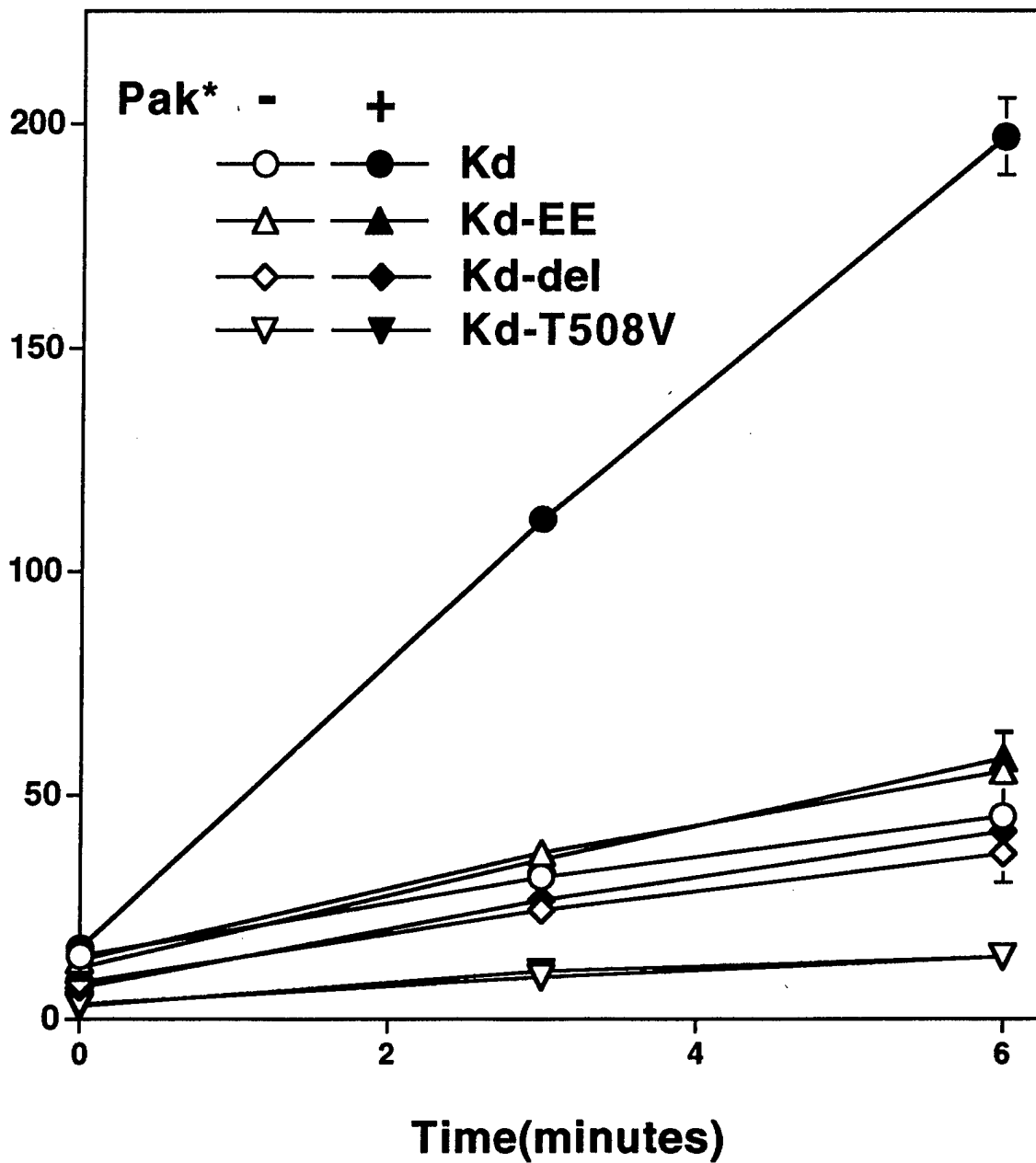
Fig. 6. Inhibition of H83,86L Pak-induced cytoskeletal changes by dominant negative D460N-LIM-kinase. BHK cells expressing H83,86L Pak and LIM kinase were identified by double immunofluorescence as described in Methods. Phalloidin-stained actin cytoskeletal changes are shown in cells expressing H83,86L Pak and LIM-kinase. A and B: Large dorsal ruffles, thickening of peripheral actin cables and aggregates of actin are present in cells expressing H83,86L Pak. C: LIM-kinase enhanced actin aggregation. D: Inhibition of dorsal ruffles and actin aggregation by coexpression of D460N-LIM-kinase with H83,86L Pak. Arrowheads show cells expressing H83,86L-Pak and arrows show cells expressing H83,86L-Pak plus WT-or D460N-LIM-kinase. **E: Catalytically-inactive H83,86L Pak (K299R) induced some irregular ruffles but no large dorsal ruffles or actin aggregates.** **F: Co-expression of D460N LIM-kinase did not block the effects of catalytically-inactive H83,86L Pak (K299R).** G: Distribution of actin cytoskeletal phenotypes in the population of cells expressing the indicated proteins. Fifty to one-hundred cells per transfection were scored in a single blind manner by 2 independent observers. Each transfection was repeated 3 times with similar results.

Fig 7. Colocalization of Pak and LIM kinase with actin at membrane ruffles Cos 7 cells were transfected with WT-LIM-kinase and myc-tagged WT-Pak. The cells were then stained for LIM-kinase (rhodamine), Pak (AMCA-S) and F-actin (phalloidin) as indicated in Methods. Staining legend in bottom left corner indicates composite colors from the overlap of any two or all three fluorescent markers.

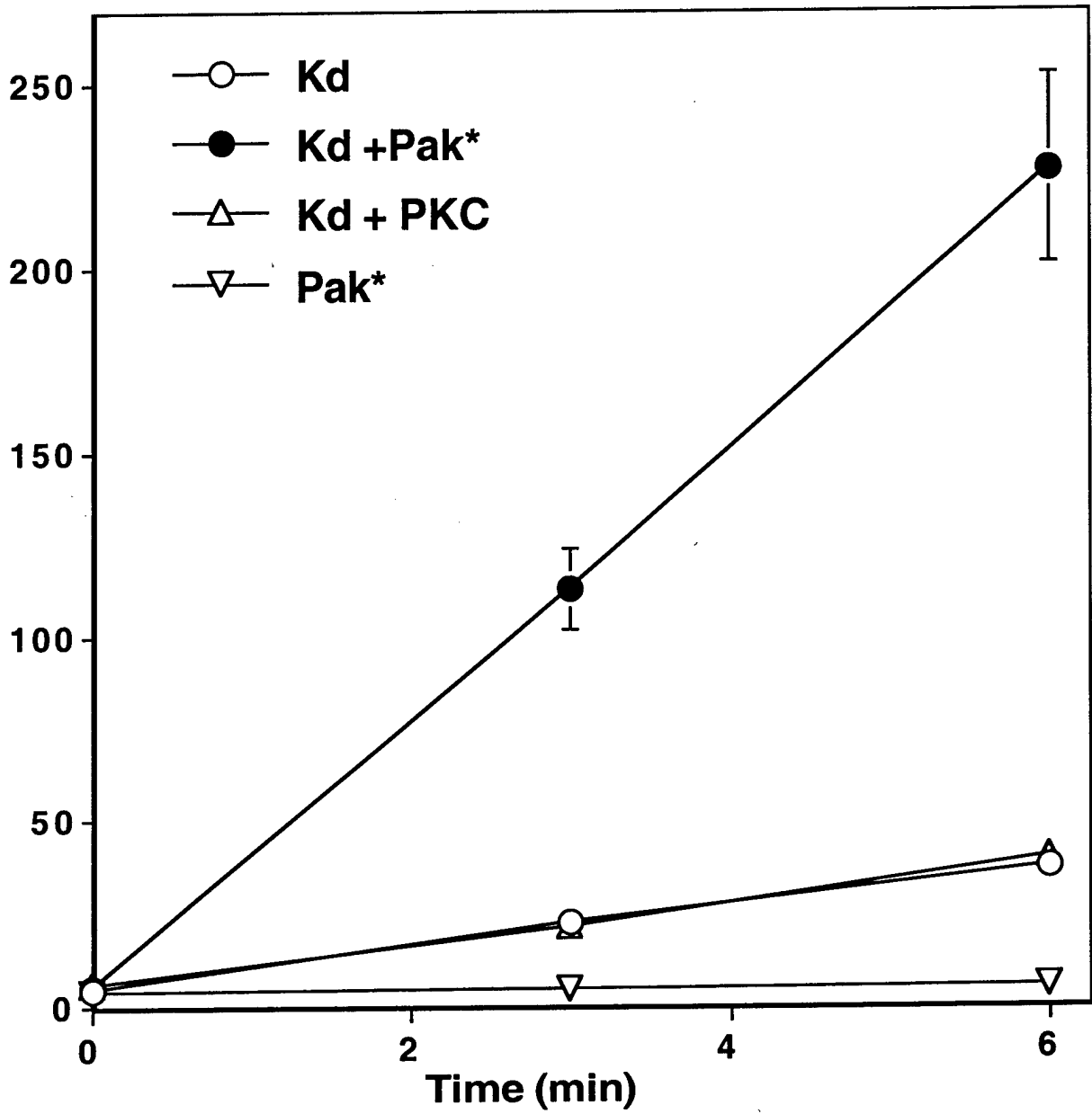
1A



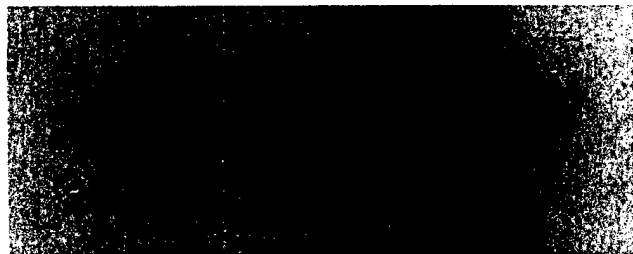
1B



1C



Pak - +



← Kd

3A

Rac-Q61L	+	-	+	+	+	+
Rac-T17N	-	+	-	-	-	-
Pak	+	+	-	-	+	-
Pak-H83,86L	-	-	+	-	-	-
WT-LIMK	+	+	+	+	-	+
Kd3	-	-	-	-	+	-



myc-Pak

IP: anti-LIM-kinase
Blot: anti-myc



LIM-kinase

IP: anti-LIM-kinase
Blot: anti-LIM-kinase



myc-Pak

Blot: lysate



myc-Rac

4A

+ Rac

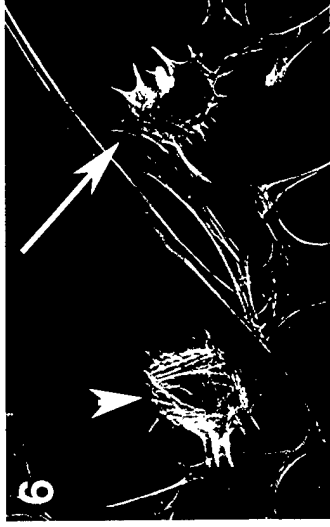
+ cdc42



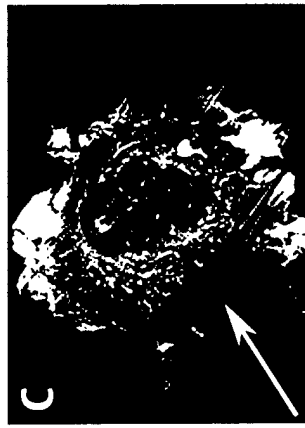
-



+LIMK



+ D460N



D460N-
LIM Kinase

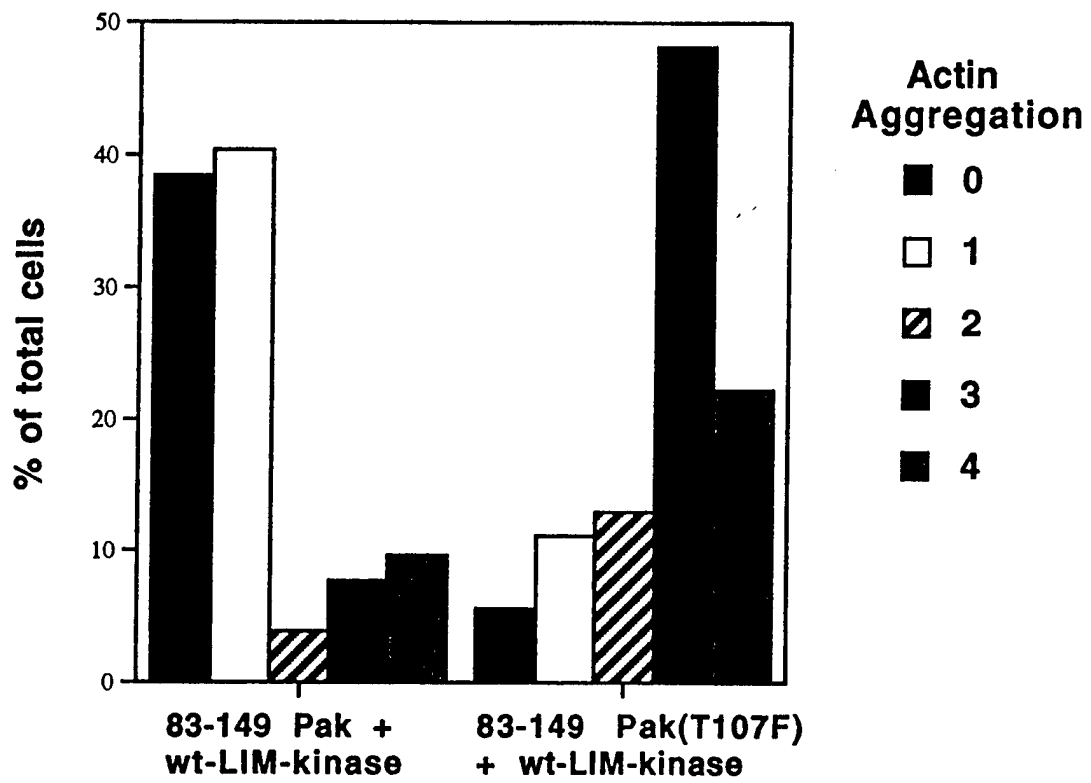
LIM Kinase
+ 83-149 Pak

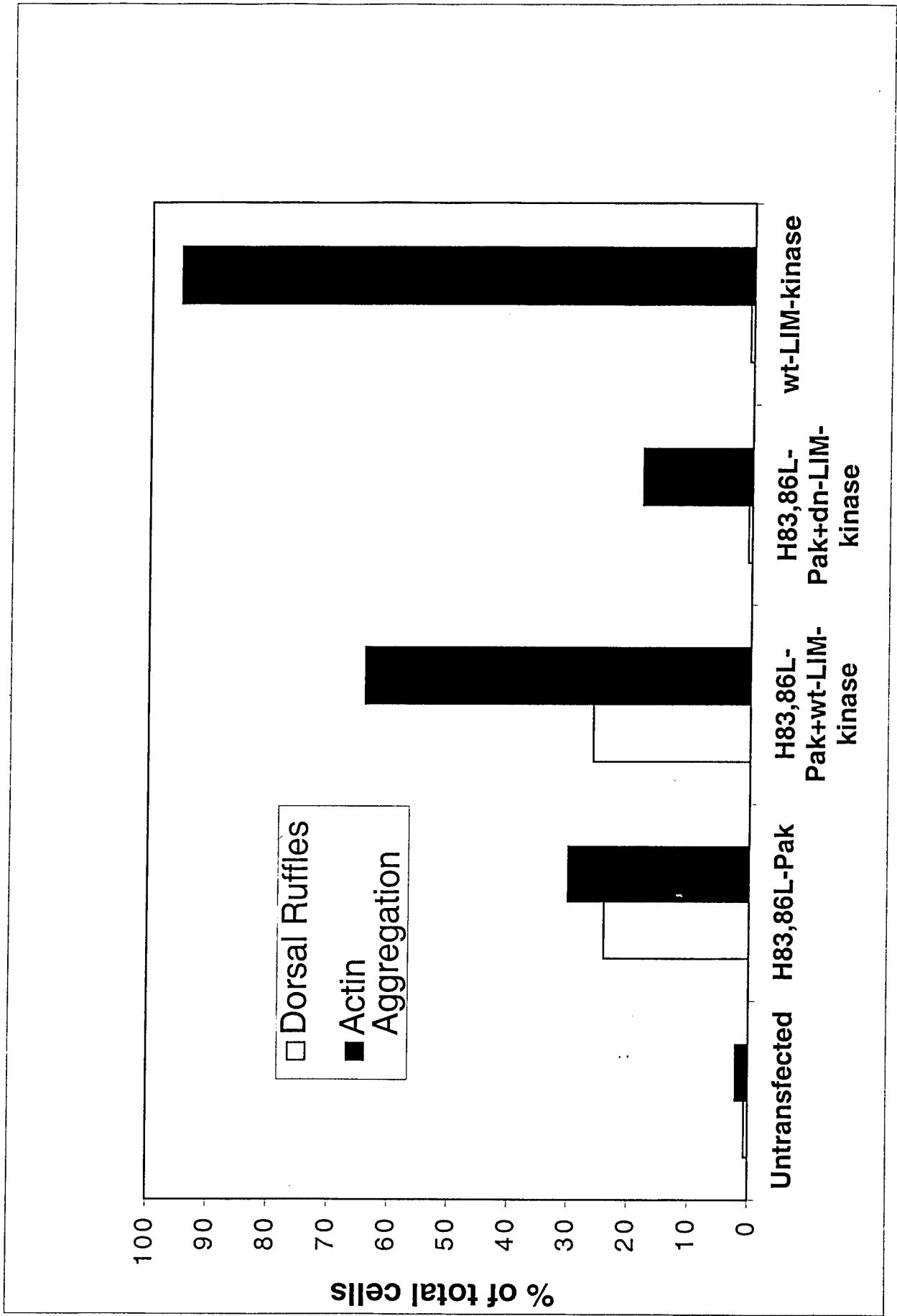
83-149 Pak

-

LIM Kinase

LIM Kinase
+
83-149(L107F)
Pak

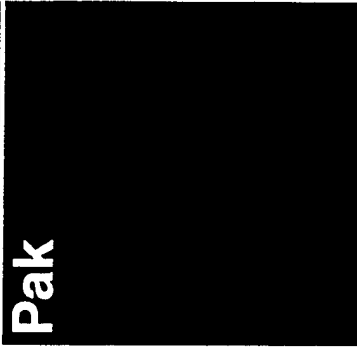




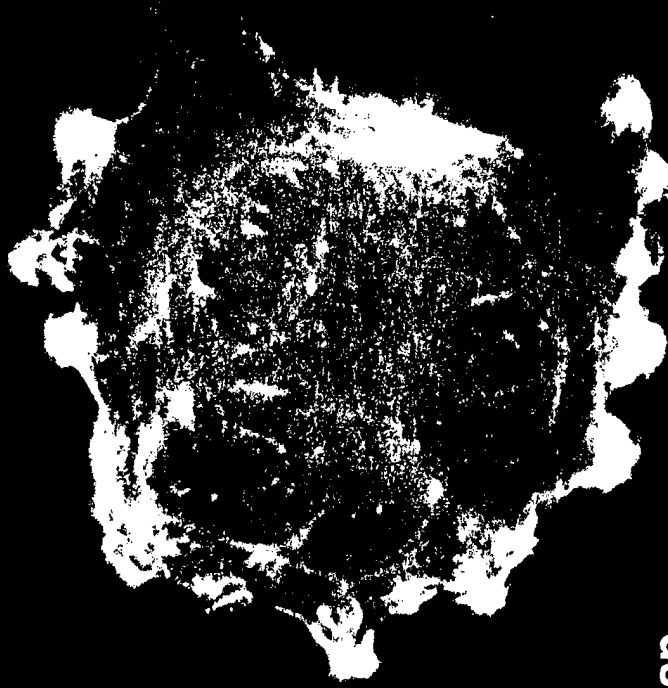
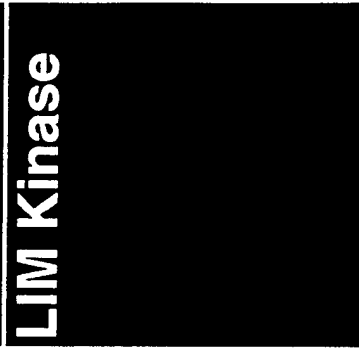
Actin



Pak



LIM Kinase



**Overlap
Legend**

