Cytoskeletal Changes Regulated by the PAK4 Serine/Threonine Kinase Are Mediated by LIM Kinase 1 and Cofilin*

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PAK4 is the most recently identified member of the PAK family of serine/threonine kinases. PAK4 differs from other members of the PAK family in sequence and in many of its functions. Previously, we have shown that an important function of this kinase is to mediate the induction of filopodia in response to the Rho GTPase Cdc42. Here we show that PAK4 also regulates the activity of the protein kinase LIM kinase 1 (LIMK1). PAK4 was shown to interact specifically with LIMK1 in binding assays. Immune complex kinase assays revealed that both wild-type and constitutively active PAK4 phosphorylated LIMK1 even more strongly than PAK1, and activated PAK4 stimulated LIMK1’s ability to phosphorylate cofilin. Immunofluorescence experiments revealed that PAK4 and LIMK1 cooperate to induce cytoskeletal changes in C2C12 cells. Furthermore, dominant negative LIMK1 and a mutant cofilin inhibited the specific cytoskeletal and cell shape changes that were induced in response to a recently characterized constitutively activated PAK4 mutant.

Regulation of the organization of the actin cytoskeleton is critical to mammalian cell function. Cytoskeletal organization plays an important role in most cellular activities including cell motility, adhesion, proliferation, and cytokinesis (1). Signaling enzymes that regulate the cytoskeleton can have adverse effects when they are improperly regulated and can lead to abnormalities including oncogenic transformation and defects in cell proliferation and motility (2–7). Understanding the mechanism by which cytoskeletal regulatory proteins regulate polymerized actin structures is thus of critical biological importance. Members of the Rho family of small GTPases have key roles in regulating the organization of the actin cytoskeleton in mammalian cells. Three of the major players of this family, Cdc42, Rac, and Rho, were shown to induce the production of filopodia, lamellipodia, and stress fibers, respectively, in a variety of cell types including fibroblasts (8–10). In addition to cytoskeletal organization, the GTPases have other functions including regulation of cell adhesion, activation of signal transduction pathways, regulation of cell proliferation, oncogenic transformation, and invasiveness (8–18).

Members of the PAK family of serine/threonine kinases interact directly with GTP-loaded Rac and Cdc42 through a GTPase binding domain (referred to as a GBD or CRIB domain) (17, 19–25). PAK1 and -2 and the closely related γPAK have been reported to induce a variety of different types of cytoskeletal changes, depending on the cell type. The activities that have been attributed to the PAKs include the induction of filopodia and membrane ruffles (26), the dissolution of stress fibers, down-regulation of focal adhesions, and cell retraction (27, 28). Some of these changes are independent of the PAKs’ kinase activities or their ability to bind to the Rho GTPases (26). PAK1 was recently found to regulate the activity of another serine/threonine kinase, LIM kinase 1 (LIMK1) (29). LIMK1 is highly expressed in the brain, although it is also expressed in a wide variety of other tissues. A defect in LIMK1 is linked to the neurological disorder Williams syndrome, and may prevent neurons from making their proper connections (30). LIMK1 causes dramatic cytoskeletal changes, primarily the induction of actin clusters. LIMK1 operates by phosphorylating and inactivating another cytoskeletal regulatory protein, cofilin, thereby inhibiting cofilin’s actin depolymerization activity (31, 32). Activated Rac also leads to the phosphorylation of LIMK1, and PAK1 may serve as a link between Rac and LIMK1 phosphorylation (29, 31, 33).

The most recent PAK family member to be identified is PAK4 (25). PAK4 differs significantly from the other PAKs in sequence, especially in its regulatory domain, although it does have a modified GBD. PAK4 was first identified as a target of Cdc42, which forms a link between Cdc42 and filopodia formation. While wild-type PAK4 on its own does not induce dramatic cytoskeletal changes, co-expression of PAK4 with Cdc42 potentiates and prolongs the induction of filopodia by Cdc42. In contrast to PAK1 (26), the ability of PAK4 to regulate cytoskeletal organization is strictly dependent on its kinase activity and on the presence of its GBD (25). We have recently characterized a constitutively active PAK4 mutant, PAK4(S445N). PAK4(S445N) leads to a transient induction of filopodia in fibroblasts that can be seen within minutes after cells are plated onto fibronectin coated surfaces. More long term effects include cell rounding, dissolution of stress fibers, reduced production of focal adhesions, and inhibition of cell spreading (34). Many of these effects of PAK4(S445N) are similar to those observed in response to activated Cdc42 or Rac.

Here we have further characterized the function of PAK4 and PAK4(S445N) in the myoblast cell line C2C12. We have found that in C2C12 cells, PAK4(S445N) also induced rounding and dissolution of stress fibers. Instead of stress fibers, C2C12...
cells expressing high amounts of PAK4(S445N) often had aggregates of polymerized actin. Since these appeared similar to the actin clusters that are induced by LIMK1, this led us to investigate whether PAK4 may also signal through LIMK1. We have found that PAK4 and PAK4(S445N) both strongly phosphorylated LIMK1. In fact, they phosphorylated LIMK1 more efficiently than equivalent amounts of wild-type or constitutively active PAK1. Activated PAK4 also increased the ability of LIMK1 to phosphorylate cofilin. The induction of actin clusters by LIMK1 was potentiated in the presence of PAK4 or PAK4(S445N), while a dominant negative LIMK1 inhibited PAK4(S445N)-induced cytoskeletal changes. Likewise, a non-phosphorylatable cofilin mutant blocked the cytoskeletal and cell shape changes induced by PAK4(S445N).

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Myc-tagged PAK4 and PAK4(K350M) are described in Ref. 25. cDNA encoding PAK4 was subcloned into the XhoI and SmaI sites of pIRE2-EGFP vector (CLONTECH). Constitutively active Myc-PAK4(S445N) in pCAN and in pIRE2-EGFP are described in Ref. 34. and contain a serine to glutamate substitution at amino acid 474 and a serine to asparagine substitution at amino acid 445 (34). pFASTBAC/HindIII, pFASTBAC/EcoRI and pFASTBAC/HindIII sites of pFASTBAC/HTb vector, a baculovirus expression vector from BAC-TO-BAC Baculovirus Expression Systems (Life Technologies, Inc.). As described in Ref. 31, cDNA encoding LIMK1 was subcloned into pCDNA expression vector containing a Myc epitope tag, and cDNA encoding LIMK1M1 (a kinase-inactive mutant of LIMK1 containing the D446E mutation) was subcloned into pCS2 expression vector containing six Myc epitope tags. cDNA encoding LIMK1 was also subcloned into pEBG, a eukaryotic GST expression vector, to obtain LIMK1 fused with GST. To generate pFASTBAC1/GST-LIMK1 and pFASTBAC1/GST-LIMK1M1, cDNA encoding GST was generated by polymerase chain reaction using pGEX-2T (Amersham Pharmacia Biotech) as a template. The GST cDNA was then subcloned into the BamHI site of pFASTBAC1, a baculovirus expression vector from BAC-TO-BAC Baculovirus Expression Systems (Life Technologies), to construct the pFASTBAC1/GST vector. cDNAs encoding LIMK1 and LIMK1M1 were then subcloned into the XhoI and HindIII sites of pFASTBAC1/GST to generate pFASTBAC1/GST-LIMK1 and pFASTBAC1/GST-LIMK1M1. Myc-tagged cofilin and cofilin(S3A) (31) were subcloned into the pCDA vector. cDNA encoding cofilin was also subcloned into the pGEX bacterial expression vector to generate a GST-cofilin vector (31). PAK1 and PAK1(T423E) were gifts from J. Chernoff.

**Antibodies and Phalloidin**—Mouse anti-Myc (sc-40) antibody is from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-PAK4 antibody (E40–883) was generated against full-length human PAK4 protein (purified using the Life Technologies BAC-TO-BAC Baculovirus Expression System in conjunction with Pharmingen). Rab- bit polyclonal anti-LIMK1 antibody is from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-mouse IgG conjugated with Alexa 350 is from Molecular Probes, Inc. (Eugene, OR). Rhodamine-conjugated phalloidin is from Molecular Probes.

**Cell Culture and Transfection**—Sf9 insect cells were grown at 27 °C, and cultured in TC-100 medium containing 10% heat-inactivated fetal bovine serum (Sigma). All other cells were grown at 37 °C in 5% CO2. 293 and C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Life Technologies). NIH3T3 cells were cultured in Dulbecco’s Modified Eagle’s medium containing 10% donor bovine calf serum (JRH Biosciences). For transfection in Sf9 cells, 1 µg of recombinant bacmid DNA was transfected into the cells in 35-mm wells (9 × 10^6 cells/well) using 6 µl of CellFectin (Life Technologies) according to the BAC-TO-BAC Baculovirus Expression System (Life Technologies) instruction manual. For transfections in 293 cells, 10 µg of total DNA was transfected into the cells in 10-cm plates (50% confluent) using the calcium phosphate precipitation method. For C2C12 cells, 16 µg of total DNA was transfected into the cells in 35-mm wells (seeded with 2.5 × 10^6 cells/well) containing coverslips, using the calcium phosphate precipitation method. For NIH3T3 cells, 2 µg of total DNA was transfected into the cells in 35-mm wells (3 × 10^5 cells/well).
using the LipofectAMINE (Life Technologies) method according to the manufacturer’s protocol.

**Protein Binding Assays**—To investigate the interactions between PAK4 and LIMK1, equal amounts of purified recombinant PAK4 were incubated either alone or with an equivalent amount of purified recombinant LIMK1 (without the GST tag). The complexes were then incubated with rabbit anti-LIMK1 antibody (Upstate Biotechnology) and protein A-Sepharose at 4°C in a buffer containing 20 mM Tris-Cl (pH 7.6), 100 mM NaCl, 0.01% Triton X-100, 1 mM Na3VO4, 20 mM glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine. After washing twice with PBS, protein complexes associated with the beads were analyzed by SDS-PAGE and Western blot using a mouse monoclonal antibody against PAK4 (E40–883).

**Western Blots**—Western blots were carried out as described (25).

**Protein Kinase Assays**—To assay LIMK1M1 or histone H4 (HH4) phosphorylation by the PAKs, 293 cells were transfected with Myc-PAK4 or PAK1 (wild-type or constitutively active) expression vectors. Cells were harvested in M2 buffer (35) 48 h after transfection. The Myc-tagged proteins were then immunopurified from 100 μg of cell extracts using antibody generated against the Myc epitope tag (Santa Cruz Biotechnology; c-Myc (9E10), sc-40), in M2 buffer at 4°C for 2 h to overnight. The immunoprecipitates were washed twice in M2 buffer and twice in a buffer containing 20 mM HEPES (pH 7.5) and 10 mM MgCl2. Equal amounts of immunoprecipitates were then incubated together with either immunopurified Myc-LIMK1M1 (immunopurified from 293 cell lysates as described above for the PAK proteins), purified GST-LIMK1M1, or HH4. Immune complexes and substrates were then incubated in a buffer containing 20 mM HEPES (pH 7.5), 10 mM MgCl2, 20 mM β-glycerophosphate, 10 mM Na3VO4, 20 mM ATP, and 5 μCi of [γ-32P]ATP for 20 min at 30°C. The reaction was terminated with SDS-PAGE sample buffer, followed by SDS-PAGE and autoradiography.

To analyze the kinase activity of wild-type GST-LIMK1, NIH3T3 cells were transfected with pEBG-LIMK1 (a eukaryotic GST vector) in the absence or presence of Myc-PAK4(S445N). Cells were harvested 48 h after transfection, and the amount of GST-LIMK1 in cell lysates was normalized by Western blot. Equal amounts of GST-LIMK1 were then purified from ~100 μg of cell lysates using glutathione-conjugated agarose beads in M2 buffer at 4°C for 2 h to overnight. The agarose beads were then washed twice in M2 buffer, washed twice in a buffer containing 20 mM HEPES (pH 7.5) and 10 mM MnCl2, and then incubated in a buffer containing 20 mM HEPES (pH 7.5), 10 mM MnCl2, 150 mM KCl, 10 mM MnCl2, 20 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate, 1 mM DTT, 50 μM Na3VO4, 20 μM ATP, and 5 μCi of [γ-32P]ATP, together with 4 μg of purified recombinant GST-cofilin for 20 min at 30°C. The reaction was terminated with SDS-PAGE sample buffer, followed by SDS-PAGE and autoradiography and PhosphorImager analysis.

**Preparation of Purified GST-cofilin**—250 ml of LB/Amp was inoculated with DH5α strain Escherichia coli that was transformed with pGEX-GST-cofilin. GST-cofilin was purified using the same method to purify GST-c-Jun as described in Ref. 36.

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**Fig. 3.** LIMK1 mediates PAK4-induced cytoskeletal changes. A, C2C12 cells were transfected with the indicated expression vectors. PAK4 and PAK4(S445N) were in pIRES2-EGFP expression vectors, and LIMK1 and LIMK1M1 were Myc-tagged. PAK4 or PAK4(S445N) were identified 48 h after transfection by fluorescence microscopy to analyze GFP-expressing cells (green). Cells expressing Myc-LIMK1 and Myc-LIMK1M1 were identified by immunostaining with mouse anti-Myc antibody and goat anti-mouse antibody conjugated with Alexa 350 (blue), 48 h after transfection. Cells were stained for F-actin using rhodamine-conjugated phalloidin (red). An intense orange stain is occasionally seen when highly concentrated polymerized actin clusters are present, such as in h. Transfected cells are indicated by arrows. B, C2C12 cells were transfected with the indicated combinations of plasmids and analyzed as described for A.
and dialyzed in a buffer containing 20 mM Tris (pH 7.6), 20% glycerol, LIMK1M1 was eluted with 50 mM Tris (pH 8.0) and 200 mM glutathione with 1.2 ml of glutathione-conjugated agarose beads. For GST-GST-LIMK1M1 and GST-LIMK1 lysates were pooled and incubated diazoyl in a buffer containing 20 mM Tris (pH 8.0), 1 mM DTT, and 6 units of bovine thrombin (Sigma; T9677) overnight at 4 °C. The mixture was then centrifuged, and the supernatant was transferred to a new tube. The beads were then incubated with high salt/DTT buffer containing 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 5 mM MgCl₂, 1 mM DTT, 1% Nonidet P-40 for 30 min at 4 °C. The supernatant was collected following centrifugation. The two supernatants were pooled and dialyzed in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, and 0.1 mM DTT. The protein was then analyzed by SDS-PAGE to ensure that it was the correct size and the GST had been removed.

Immunofluorescence Microscopy—48 h after transfection, C2C12 cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.1% Triton X-100, and blocked with 5% goat serum for 20 min at room temperature. Cells were stained for the presence of Myc-LIMK1, Myc-LIMK1M1, Myc-cofilin, or Myc-cofilin(S3A) with mouse anti-Myc antibody and goat anti-mouse IgG conjugated with Alexa 350 (blue) (Molecular Probes). Filamentous actin was visualized by staining the cells with rhodamine-conjugated phallolidin (red). The presence of pIRES2-EGFP-PAK4 or pIRES2-EGFP-PAK4(S445N) was detected by fluorescence microscopy to visualize GFP-expressing cells (green). The images were taken with a Nikon DIAPHOT 300 inverted microscope with epifluorescence attachments and a color CCD camera.

RESULTS
PAK4 Interacts with LIMK1—To determine whether PAK4 and LIMK1 interact with each other, purified recombinant PAK4 was incubated alone or together with purified LIMK1. The samples were then incubated with anti-LIMK1 antibody and protein A-Sepharose beads. The complexes were then washed and analyzed by Western blot. The presence of PAK4 in the complexes was assessed by probing the blot with anti-PAK4 antibody (see Fig. 1). The results indicate that PAK4 interacts specifically with LIMK1.

PAK4 Phosphorylates LIMK1 and Stimulates Its Ability to Phosphorylate Cofilin—To determine whether PAK4 phosphorylates LIMK1, 293 cells were transfected with wild-type PAK4, activated PAK4(S445N), kinase-defective PAK4(K350M), wild-type PAK1, or activated PAK1(T423E), all fused to Myc epitope tags. After transient transfection, immunopurified PAK4 or PAK1 was incubated with equal amounts of either unimmunopurified Myc-LIMK1M1(D446E), purified GST-LIMK1M1(D446E), or HH4. LIMK1M1(D446E) has a point mutation in the kinase domain that abolishes its kinase activity, but it can still be phosphorylated by upstream activators (31). An immune complex kinase assay was then carried out in the presence of [γ-32P]ATP. The results indicate that LIMK1M1 was phosphorylated by wild-type PAK4, and it was phosphorylated even more strongly by PAK4(S445N) (Fig. 2A). In contrast, phosphorylation of LIMK1M1 by wild-type PAK1 was not detectable, and constitutively active PAK1(T423E) phosphorylated LIMK1M1 inefficiently compared with PAK4. (A faint band can be seen in lane 6, where purified GST-LIMK1M1 was used as a substrate.) In fact, phosphorylation of LIMK1M1 by PAK1(T423E) could only really be detected when the gel was overexposed relative to PAK4 activity (data not shown). The same amount of PAK1 and PAK1(T423E), however, phosphorylated HH4 as efficiently as PAK4 (Fig. 2A). All of the different PAK proteins were expressed at equal levels as detected by Western blot (Fig. 2A, bottom panel).

LIMK1 is known to phosphorylate the cytoskeletal regulatory protein cofilin (31, 32). To determine whether activated PAK4 increases the ability of LIMK1 to phosphorylate cofilin, NIH3T3 cells were transfected with Myc-PAK4(S445N) expression vector together with GST-LIMK1 vector. After transient expression, equal amounts of GST-LIMK1 were isolated from cell lysates using glutathione-agarose beads. GST-LIMK1 com-
plexes were then used to phosphorylate recombinant cofilin in an in vitro kinase assay in the presence of \([\gamma-32P]\)ATP. Although LIMK phosphorylated cofilin on its own, the presence of PAK4(S445N)-stimulated LIMK1 induced phosphorylation of cofilin ~4-fold (Fig. 2B).

**LIMK1 Mediates PAK4-induced Cytoskeletal Changes**—To see whether PAK4 and LIMK1 cooperate to induce cytoskeletal changes, C2C12 cells were transfected with PAK4 (wild-type or activated)-IRE2-EGFP vector or Myc-tagged LIMK1 (wild-type or kinase-inactive) expression vectors, alone or in combination. 48 h after transfection, cells were stained with rhodamine-conjugated phalloidin (red) in order to visualize polymerized actin structures. PAK4-IRE2-EGFP-expressing cells were identified by fluorescence microscopy (green), and cells expressing LIMK1 were detected by immunofluorescence using anti-Myc antibody and Alexa 350 conjugated secondary antibody (blue). Representative phalloidin-stained cells are shown in Fig. 3, A and B. Transfected cells are indicated by an arrow. Fig. 3A shows cells that were transfected with either PAK4, activated PAK4(S445N), LIMK1, or kinase-dead LIMK1M1 expression vectors. While expression of wild-type PAK4 had little effect on the actin cytoskeleton (Fig. 3A, a and b), PAK4(S445N) induced dramatic cell changes, which varied depending on the expression level. In ~50% of the cells, relatively low levels of PAK4(S445N) were expressed (as assessed by the intensity of the green color). These cells exhibited the dissolution of actin stress fibers and the formation of actin filaments at the periphery of the cell, similar to the results seen previously in fibroblasts (34) (Fig. 3A, c and d). The rest of the transfected cells, in which higher amounts of PAK4(S445N) were expressed, were rounded, lacked stress fibers, and contained actin clusters instead of stress fibers (Fig. 3A, e and f). Filopodia were generally not visible in cells expressing PAK4(S445N). This is consistent with results in Rat1 fibroblasts in which filopodia were only visible transiently after PAK4(S445N)-expressing cells were plated onto fibronectin-coated surfaces and could not be seen 48 h later (34). LIMK1 expression, as reported previously (31, 32), led to an increase in actin clusters within the cell, similar to PAK4(S445N), but unlike PAK4(S445N), it did not induce cell rounding (Fig. 3A, g and h). In contrast, LIMK1M1 led to a reduction of polymerized actin within the cell (Fig. 3A, i and j).

**Fig. 3B** shows cells that were transfected with PAK4-IRE2-EGFP or PAK4(S445N)-IRE2-EGFP vectors together with either Myc-tagged LIMK1 or Myc-tagged LIMK1M1, which was used as a dominant negative mutant. Co-transfection of PAK4 and LIMK1 resulted in an increase in polymerized actin structures (Fig. 3B, a–c). The amount of polymerized actin was generally higher than that seen with LIMK1 alone. Furthermore, cells were more rounded and less well spread than the cells containing LIMK1 alone, although not as rounded as the cells containing PAK4(S445N) (Compare with Fig. 3A, h or f). In contrast, cells transfected with PAK4 together with LIMK1M1 (Fig. 3B, d–f) did not show an increase in polymerized actin or changes in cell spreading. Cells transfected with PAK4(S445N) together with LIMK1 vector (Fig. 3B, g–i) showed a dramatic increase in polymerized actin. Polymerized actin appeared to fill the entire cells, which were rounded and condensed. Organized stress fibers, however, were not detected. The polymerized actin structures were much more abundant in cells co-expressing PAK4(S445N) and LIMK1 than in the presence of either one alone (compare with Fig. 3A, f or h). Strikingly, cells co-transfected with PAK4(S445N) and LIMK1M1 appeared similar to nontransfected cells (Fig. 3B, j–l). Virtually all of the transfected cells completely lost the polymerized actin aggregates normally associated with PAK4(S445N), even when high levels of PAK4(S445N) were expressed as detected by fluorescence microscopy. In a significant proportion of the cells, cell rounding was also diminished. The major cellular changes seen in cells expressing PAK4(S445N) alone or together with LIMK1 or LIMK1M1 are summarized later in Fig. 5. Taken together, these results indicate that PAK4 and LIMK1 can cooperate to induce actin clusters, and dominant negative LIMK1 inhibits the cell shape and cytoskeletal changes induced by activated PAK4.

**Cofilin Is Necessary for PAK4-induced Cytoskeletal and Cell Shape Changes**—LIMK is thought to function by phosphorylating cofilin and thereby inhibiting its ability to depolymerize actin (31). To see whether cofilin mediates the cytoskeletal changes induced by PAK4, C2C12 cells were transfected with Myc-tagged cofilin or a mutant Myc-tagged cofilin(S3A) (which cannot be phosphorylated by LIMK1), either alone or together with PAK4(S445N)-IRE2-EGFP vector (Fig. 4). The right four panels show polymerized actin staining in cells as visualized by
staining with rhodamine-conjugated phalloidin. The left four panels show fluorescence micrographs of the same fields, used to identify the cells that contain the expressed plasmids, as described for Fig. 3. Cells expressing cofilin had a reduction in polymerized actin (Fig. 4, a and b), as expected because cofilin acts to depolymerize filamentous actin (37). Cells expressing cofilin(S3A) showed a more dramatic reduction in polymerized actin than the wild-type cofilin (Fig. 4, c and d). This is consistent with the idea that cofilin(S3A) is a deregulated form of cofilin that can no longer be inhibited by phosphorylation (29). Cells expressing PAK4(S445N) (Fig. 4, e and f) were rounded with an increase in polymerized actin structures as seen in Fig. 3. Similar to LIMK1M1, co-expression of cofilin(S3A) with PAK4(S445N) (Fig. 4, g and h) led to a near complete loss of polymerized actin as well as a loss of the rounded morphology typically induced by PAK4(S445N) (compare f and h). The major cellular changes seen in cells expressing PAK4(S445N) alone or together with the cofilin mutant or LIMK1 are summarized in Fig. 5. In summary, these results indicate that cofilin is an important mediator of the cytoskeletal and cell shape changes that are induced by activated PAK4.

DISCUSSION

Members of the PAK family have been shown to regulate a wide variety of cytoskeletal changes, usually by mediating responses to Rho family GTPases. Here we have shown that PAK4 phosphorylates the serine/threonine kinase LIMK1 and stimulates its activity. Previous work has shown that recombiant constitutively active PAK1 phosphorylates purified LIMK1 and that PAK1 may be the link between Rac and LIMK1 (29). Our phosphorylation assays were carried out using PAK proteins that were expressed in mammalian cells and immunopurified from cell lysates rather than recombiant proteins. When we compared immunopurified PAK1 with immunopurified PAK4, PAK4 phosphorylated LIMK1 significantly more strongly than an equivalent amount of activated PAK1, and wild-type PAK1 did not phosphorylate LIMK1 at all. These differences in substrate specificity are not surprising, because PAK1 and PAK4 are quite different in sequence and function (25). In fact, PAK4 and PAK1 share only ~50% sequence identity in the kinase domain and no identity in the regulatory domain except for the GBD motif (25).

LIMK1 is known to regulate the organization of the actin cytoskeleton by inhibiting the activity of the actin-depolymerizing protein cofilin (31). The exact function of LIMK1 however, appears to be complex, since it has been shown to mediate diverse types of morphological changes in response to different activators. For example, LIMK1 is regulated by Rac (29), a GTPase that can both promote lamellipodia formation (9) and trigger a decrease in stress fiber formation in some cells (38). Similarly, we have shown that LIMK1 is a target for PAK4, which also causes a decrease in stress fiber formation (Fig. 3).2 However, LIMK1 has also been shown to be activated by RhoA (39, 40), a GTPase that triggers the formation of stress fibers (10). These seemingly contradictory results may be partly explained by the fact that the formation and dissolution of stress fibers are regulated not only by actin polymerization and depolymerization but also by phosphorylation and dephosphorylation of myosin light chain, an important component of stress fibers (39–41). The mechanism by which LIMK1 may contribute to the production of such diverse types of cytoskeletal structures in response to divergent types of activators is thus likely to involve the coordinated regulation of different types of cytoskeletal regulatory proteins acting in concert with LIMK1.

Our results suggest that the function of PAK4 is also more complex than we previously predicted. While we and others have found evidence for a role for PAK4 and other PAK family members in cytoskeletal organization in response to Rac and Cdc42, some of the cytoskeletal changes induced by activated PAK4, such as actin clustering and cell rounding, differ from Rac- or Cdc42-induced changes. It seems likely, therefore, that PAK4 may regulate cytoskeletal changes in response to both Rho GTPase-dependent and -independent stimuli. It is interesting that dominant negative LIMK1 inhibits most of the changes induced by PAK4(S445N) including cell rounding and the formation of actin clusters, which is consistent with the idea that cell shape and cytoskeletal organization are tightly linked. Currently, we do not know the physiological significance of the actin clusters that are induced by activated PAK4(S445N). Since LIMK1 induces similar structures, they may be a result of the increase in actin polymerization triggered by inhibition of cofilin activity.

LIMK1 was originally shown to be activated by the Rho GTPase Rac (29, 31, 32), and some studies have shown that it is also activated by Rho (39, 40). It is not activated by Cdc42, however, and it is not required for the induction of filopodia by Cdc42 (29, 32). This raises questions about the role for PAK4 in LIMK1 activation, since PAK4 was identified as a kinase that is preferentially regulated by Cdc42 (25). It should be noted, however, that although PAK4 binds more efficiently to Cdc42, it can also bind weakly to Rac (34), and its kinase activity is partially elevated in response to expression of activated Rac. However, recently it was shown that unlike LIMK1, LIMK2 is activated by Cdc42 (and also by Rho) (43). Furthermore, LIMK2, but not LIMK1, was shown to be required for filopodia formation triggered by Cdc42. Thus, while they both inhibit cofilin activity, LIMK1 and LIMK2 regulate different types of cytoskeletal changes. This suggests that the two kinases may operate in concert with different cytoskeletal regulatory proteins. Upstream kinases that regulate LIMK2’s activity have not yet been identified. Since LIMK2 shares considerable sequence similarity with LIMK1, an intriguing possibility is that PAK4 could actually be the link between Cdc42 and LIMK2 activation. Further work will be required to determine whether LIMK1 or LIMK2 or both are actually the physiological targets for PAK4.

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