Inhibition of Myosin Light Chain Kinase by p21-Activated Kinase

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p21-activated kinases (PAKs) are implicated in the cytoskeletal changes induced by the Rho family of guanosine triphosphatases. Cytoskeletal dynamics are primarily modulated by interactions of actin and myosin II that are regulated by myosin light chain kinase (MLCK)–mediated phosphorylation of the regulatory myosin light chain (MLC). p21-activated kinase 1 (PAK1) phosphorylates MLCK, resulting in decreased MLCK activity. MLCK activity and MLC phosphorylation were decreased, and cell spreading was inhibited in baby hamster kidney–21 and HeLa cells expressing constitutively active PAK1. These data indicate that MLCK is a target for PAKs and that PAKs may regulate cytoskeletal dynamics by decreasing MLCK activity and MLC phosphorylation.

Cytoskeletal remodeling is important in many cellular responses, including cell adhesion, spreading, and motility (*1–3*). Rho family members of small guanosine triphosphatases (GTPases)—Rho, Rac, and Cdc42—have been implicated as critical regulators of cytoskeletal changes (*4, 5*). PAK1, an effector molecule activated by Rac and Cdc42, localizes to Rac-induced membrane ruffles (*6*), and mutationally activated PAKs induce cytoskeletal rearrangement (*7, 8*), suggesting a role for PAKs in cytoskeletal dynamics. Cytoskeletal dynamics are primarily modulated by actin and myosin II (*9–11*). Because actin–myosin II interactions are regulated by the phosphorylation of the regulatory MLC by MLCK (*12, 13*), we examined the effects of PAK1 on MLCK activity.

Phosphorylation of Ser^{19} of the regulatory MLC stimulates the actin-activated adenosine triphosphatase (ATPase) activity of myosin II and regulates the force-generating ability of myosin II in vivo (*1, 10, 12, 13*). Although MLCK requires calcium and calmodulin for activity (*12*), phosphorylation by other protein kinases can increase (*14*) or decrease (*15, 16*) MLCK activity. Therefore, we investigated the possibility that PAK1 phosphorylates and regulates MLCK activity. PAK1 phosphorylated MLCK in vitro and caused the incorporation of ~1 mol PO_4/mol MLCK (Fig. 1B). This phosphorylation was independent of calmodulin binding to MLCK (*17*). Under conditions in which we obtained stoichiometric phosphorylation of the enzyme by PAK1, the catalytic activity of MLCK was decreased by ~50% when assayed at a saturating calmodulin concentration (Fig. 1) (*18*). This inhibition appears to be a direct effect on the maximum velocity (V_{max}) and not an effect on the affinity of MLCK for calmodulin ($K_{calmodulin}$) (*17*). This is in contrast to MLCK phosphorylation by cyclic adenosine 5′-monophosphate–dependent protein kinase and calmodulin-dependent kinase II, which change the $K_{calmodulin}$ without affecting the V_{max} (*15, 16*). Thus, the cellular effects of PAK could be mediated through the phosphorylation and inactivation of MLCK and a decrease in MLC phosphorylation.

We studied cell adhesion and cell spreading, which result in the activation of PAK (*19*). Baby hamster kidney–21 (BHK-21) cells were transfected with wild-type PAK1 (wtPAK1) or with a constitutively active PAK1 [$Thr^{423} \rightarrow Glu^{423}$ (T423E)] (*20*), and equal expression of wtPAK1 or PAK1 (T423E) was confirmed by protein immunoblot analysis. Control cells and those expressing PAK1 (T423E) were placed on fibronectin-coated coverslips and allowed to adhere and spread for 2 hours (*21*). Cells were then fixed and examined with fluorescence microscopy. Nontransfected control cells, virus controls encoding LacZ, and cells expressing wtPAK1 attached and spread normally, with 80% of the cells (n = 271) showing a well-spread morphology at 2 hours (Fig. 2A). Phalloidin staining revealed a typical fibroblastlike morphology with numerous stress fibers (Fig. 2A). Cells expressing PAK1 (T423E) attached to fibronectin normally, but cell spreading was reduced, and only 27% of the cells (n = 303) had spread in 2 hours (Fig. 2A).

Cell adhesion and cell spreading on the extracellular matrix require active cytoskeletal remodeling that is dependent on actin polymerization and the interaction of actin with myosin II (*2, 10, 22*). Actin–myosin II interactions are regulated by the phosphorylation of MLC (*13, 14*), and MLC phosphorylation on Ser^{19} increases during postmitotic cell spreading at the leading edge and returns to the baseline level in completely spread cells (*22, 23*). Furthermore, 2,3-butanedione monoxime (BDM) reversibly inhibits the ATPase activity of nonmuscle myosin II and the spreading of postmitotic cells (*22*). We confirmed the participation of myosin II in spreading by showing that BDM (20 mM) inhibits the spreading of BHK-21 cells for up to 90 min (Fig. 2B). The inhibition of cell spreading by BDM was dose-dependent over the concentration range of 2 to 50 mM (*24*). It was also reversible. The cells had spread normally 45 min after the BDM was removed (Fig. 2B). Similarly, cell spreading was inhibited by the MLCK inhibitor ML-7 (*25*), indicating that spreading requires MLCK activity (*24*).

We also analyzed the effect of PAK1 expression on MLCK activity and the phosphorylation of MLC in vivo. Control cells or those cells that expressed PAK1 (T423E) were lysed, and endogenous MLCK was immunoprecipitated and assayed for activity in the presence of a saturating concentration of calmodulin (*18*). MLCK that was immunoprecipitated from cells expressing PAK1 (T423E) showed decreased activity in comparison to that of MLCK immunoprecipitated from control cells or from cells expressing wtPAK1 (Fig. 3).

Immunoblot analysis with an antibody that specifically recognizes MLC phosphorylated on Ser^{19} (*26*) showed a gradual increase in phosphorylation during the spreading of nontransfected control cells (Fig. 4). Cells that were transfected with control plasmids (LacZ or wtPAK1) exhibited similar increases in phospho-

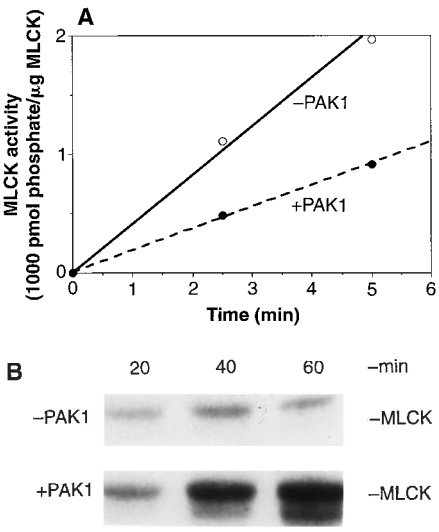

Fig. 1. (**A**) Activity of MLCK incubated with (solid circles) or without (open circles) GST PAK1. MLCK activity was determined with purified MLCs as substrate in the presence of a saturating concentration of calmodulin (*18*). The solid line shows control MLCK activity; the dashed line shows MLCK activity after PAK1 phosphorylation. (**B**) An autoradiogram of MLCK incubated alone (–PAK1) or with PAK1 (+PAK1) for 20, 40, or 60 min and then subjected to SDS-PAGE and autoradiography.

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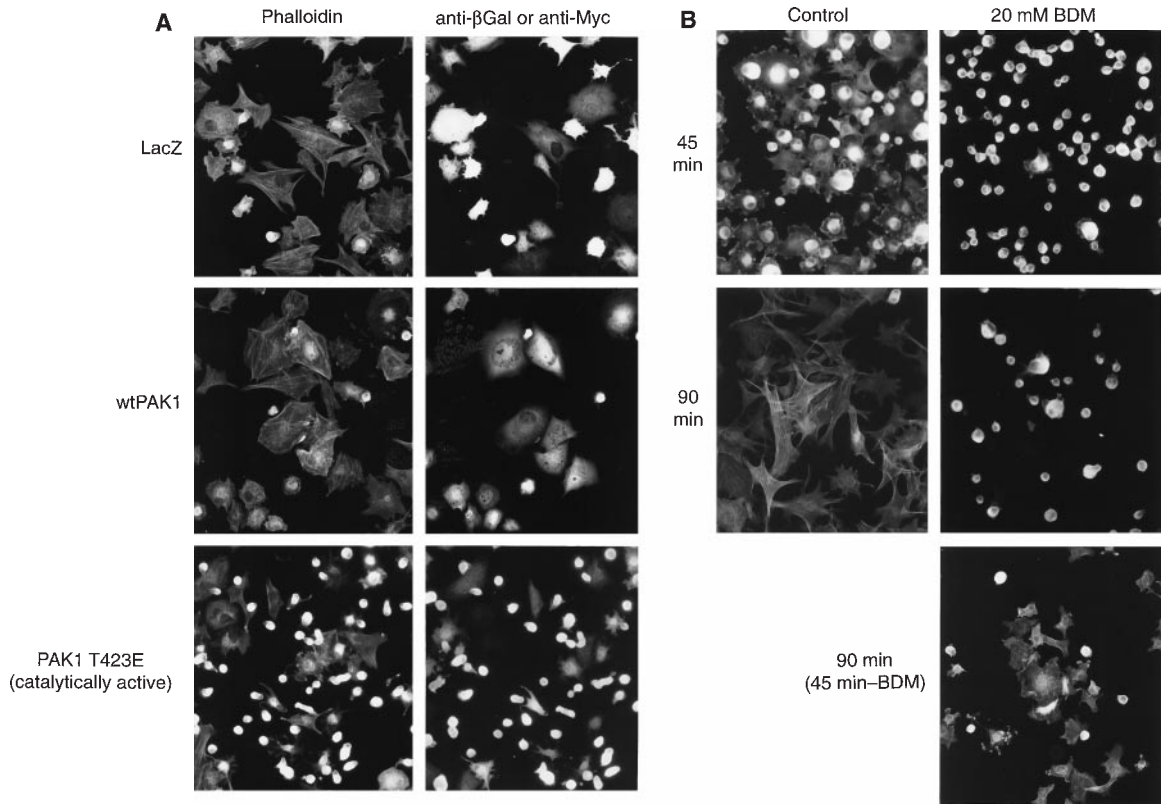

Fig. 2. Inhibition of cell spreading after expression of PAK1 (T423E) or after the addition of inhibitors of myosin ATPase activity. (**A**) Cells were transfected with LacZ, wtPAK1, or PAK1 (T423E) (*20*), allowed to express protein for 6 hours, harvested, and placed on coverslips that were coated with fibronectin (*21*). After 2 hours, the cells were fixed and stained with phalloidin (left panels), with an antibody to β-Gal (top right), or with an antibody to Myc (middle and bottom right panels) to visualize cells expressing wtPAK1 or PAK1 (T423E). (**B**) BHK-21 cells were placed on fibronectin-coated coverslips either in the presence or in the absence of 20 mM BDM (*21*). Cells were fixed after 45 or 90 min and stained with phalloidin. The bottom panel shows cells in which the BDM was washed away after 45 min and incubated for another 45 min.

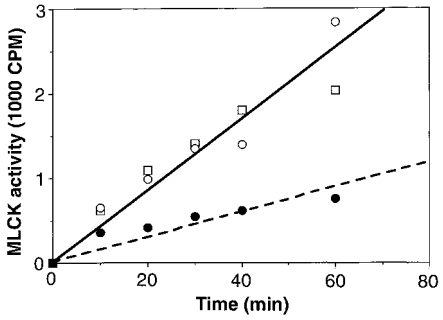

Fig. 3. Decreased MLCK activity in cells expressing PAK1 (T423E). MLCK was immunoprecipitated from nontransfected HeLa cells (open squares) or from cells transfected with wtPAK1 (open circles) or PAK1 (T423E) (solid circles). The immunoprecipitates were then assayed for MLCK activity with MLC as substrate (*18*). Solid line, MLCK activity in control cells; dashed line, MLCK activity in cells expressing PAK (T423E).

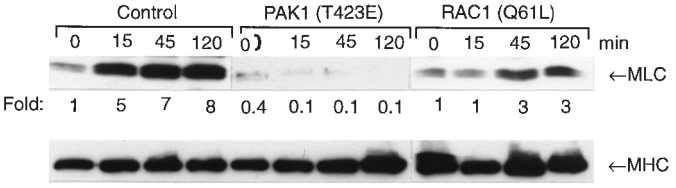

Fig. 4. Inhibition of MLC phosphorylation in BHK-21 cells. Control cells, cells expressing PAK1 (T423E), or cells expressing Rac1 (Q61L) were placed in fibronectin-coated six-well plates. At the indicated time points, cells were lysed, and MLC that was phosphorylated on Ser^{19} was detected by immunoblotting (*26*). The numbers at the bottom of each lane give the increase over the control for each data point by phosphoimager quantification. The bottom row shows that equivalent amounts of myosin were present in each sample, as determined by immunoblotting with an antibody to MHC.

rylation of MLC. In contrast, cells expressing PAK1 (T423E) showed reduced phosphorylation at all time points (Fig. 4). Consistent with the ability of activated PAK1 to decrease MLC phosphorylation, MLC phosphorylation is decreased in cells expressing Rac1 [Gln^{61} → Leu^{61} (Q61L)], the upstream activator of PAK (Fig. 4). This inhibitory effect of Rac1 (Q61L) could be partially reversed by coexpressing a PAK1 autoinhibitory domain (amino acids 83 through 149) (*24*). Thus, catalytically active PAK1 may act in vivo to inhibit MLCK activity, MLC phosphorylation on Ser^{19}, and cell spreading.

The dynamic nature of cell rearrangement and motility requires complex coordinated regulation of the cytoskeleton by Rho, Rac, and Cdc42 (*3–5, 27*). Rho kinase, the Rho effector molecule, phosphorylates myosin phosphatase and inhibits its activity (*28*) and may directly phosphorylate MLC as well (*29*). Both activities serve to increase the phosphorylation of MLC and to stimulate contractility (*28, 29*). This regulatory activity of Rho may be important in the contractile events necessary for maintaining the rigidity that is characteristic of fully spread, stationary fibroblasts (*4, 27–29*).

In contrast, Rac and Cdc42 appear to regulate actin rearrangements that are important in the early stages of cell spreading (*4*) as well as dynamic morphological changes that are associated with cell migration (*9, 10*). Our results identify MLCK, which regulates myosin II function, as a specific target for PAK. The data of Fig. 3 indicate that a decrease in MLCK activity of 50 to 60%, induced by expression of PAK1 (T423E), is sufficient to inhibit MLC phosphorylation by 80 to 90%. A change in MLCK activity of this magnitude is likely to be sufficient to account for the observed decrease in MLC phosphorylation. However, we cannot rule out the possibility that PAK also modulates other pathways that affect the steady-state level of MLC phosphorylation, although preliminary

data indicate that PAK does not stimulate MLC phosphatase activity (*30*). Thus, regulation of MLCK activity and, hence, MLC phosphorylation appears to be an important component of Rac- and Cdc42-dependent cytoskeletal remodeling in spreading cells. Because Rho kinase and PAK have opposing effects on MLC phosphorylation, the integrated cellular response to the activation of Rho, Rac, and Cdc42 may depend on the timing of GTPase activation as well as the intracellular localization and extent of MLC phosphorylation.

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18. Purified smooth muscle MLCK (5.2 μg) was incubated at 30°C for 1 hour by itself (autophosphorylation control) or with constitutively active recombinant glutathione S-transferase–PAK1 (0.5 μg) [U. G. Knaus, S. Morris, H.-J. Dong, J. Chernoff, G. M. Bokoch, *Science* **269**, 221 (1995)] at 30°C in buffer containing 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.1 mM ^{32}P-labeled adenosine 5′-triphosphate (specific activity ~2000 cpm/pmol), and 20 mM tris-HCl (pH 7.5). Aliquots were removed at various times and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. To measure MLCK activity, we incubated autophosphorylated or PAK1-phosphorylated MLCK (50 ng) at room temperature as described above, with 0.5 mM $CaCl_2$, 10^{-7} M calmodulin, 5 mM DTT, and purified regulatory MLC (10 μg). Aliquots were removed at various times and subjected to SDS-PAGE. The bands representing MLC were excised and counted. To quantify changes in MLCK activity in cells, we immunoprecipitated MLCK from nontransfected HeLa cells or from HeLa cells expressing LacZ, wtPAK1, or PAK1 (T423E) as described [P. de Lanerolle, G. Gorgas, X. Li, K. Schluns, *J. Biol. Chem.* **268**, 16883 (1993)] with affinity-purified goat antibodies to MLCK [P. de Lanerolle *et al.*, *Circ. Res.* **68**, 457 (1991)] and protein A–Sepharose. The beads were washed extensively and then assayed for MLCK activity as described. HeLa cells were used in these experiments because the MLCK antibody reacts poorly with BHK-21 MLCK on protein immunoblot analysis.
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20. The cDNAs encoding wtPAK1 or a constitutively active form of PAK1 in which Thr^{423} is mutated into Glu (T423E) were expressed in cells with the Semliki Forest virus Gene Expression System (Life Technologies, Gaithersburg, MD). The cDNAs were amplified using the polymerase chain reaction with primers that contained a Bam HI restriction enzyme site and a Myc tag at the 5′ end. These constructs were subcloned into the Bam HI site of the Semliki Forest vector pSFV3. In vitro transcription of linearized pSFV3 constructs and pSFV-Helper2 was done with SP6 RNA polymerase. RNA transfection of BHK-21 cells was performed by electroporation as described [K. Lundström *et al.*, *Eur. J. Biochem.* **224**, 917 (1994)], yielding recombinant viral stocks of ~10^7 plaque-forming units per milliliter. Viral stocks were stored at −80°C. The virus was activated per manufacturer's instructions, and BHK-21 and HeLa cells were infected in serum-free medium. Transfection efficiency of recombinant virus was routinely >95% in BHK-21 cells and >50% in HeLa cells. Cells were allowed to express protein for 6 to 8 hours after infection in serum-free medium before use in experiments.
21. For cell adhesion assays and immunofluorescence, cells were suspended in basal medium (Glasco minimal essential medium, Life Technologies) containing no serum and seeded in six-well plastic plates containing coverslips coated with fibronectin (20 μg/ml). Cells attached to coverslips were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 20 min, and then incubated with antibody to Myc (9E10) at 1:300 or with antibody to β-galactosidase (β-Gal) at 1:5000 (Promega, Madison, WI) for 1 hour. Cells were then incubated for 1 hour with rhodamine phalloidin 1:500 (Sigma) or with fluorescein isothiocyanate–conjugated antibody to mouse immunoglobulin G (IgG) 1:300 (Cappel Labs, Cochranville, PA) or with both, washed with phosphate-buffered saline, and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Slides were examined with a Nikon Labophot-ZDFX-DX epifluorescence microscope, and images were photographed with a 35-mm camera and Kodak T-MAX film. For inhibition studies, various concentrations of BDM (Sigma) and ML-7 (Calbiochem, La Jolla, CA) were added to the culture medium.
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Imaging Protein Kinase Cα Activation in Cells

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Spatially resolved fluorescence resonance energy transfer (FRET) measured by fluorescence lifetime imaging microscopy (FLIM), provides a method for tracing the catalytic activity of fluorescently tagged proteins inside live cell cultures and enables determination of the functional state of proteins in fixed cells and tissues. Here, a dynamic marker of protein kinase Cα (PKCα) activation is identified and exploited. Activation of PKCα is detected through the binding of fluorescently tagged phosphorylation site–specific antibodies; the consequent FRET is measured through the donor fluorophore on PKCα by FLIM. This approach enabled the imaging of PKCα activation in live and fixed cultured cells and was also applied to pathological samples.

For many proteins, there is a need to integrate spatial data with information on catalytic function. This is a particular concern in signal transduction processes, where the networking of multiple inputs affects the output, leading to grossly different cellular consequences. Methods applicable to the functional analysis of particular (signaling) proteins in situ would provide a significant advance in reaching a molecular description of cellular behavior.

The classical and novel protein kinase isotypes (cPKC and nPKC, respectively) undergo conformational changes in response to their second messenger, diacylglycerol (DAG) (*1*–*3*). This PKC activator is restricted to membrane compartments, and the stable membrane/DAG-associated complexes formed by PKC have traditionally provided a useful means of monitoring PKC isotype activation (*4*). However, PKC isotypes can associate with mem-

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