Uncoordinated regulation of stress fibers and focal adhesions by DAP kinase

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Summary

Death-associated protein kinase (DAP kinase) is a pro-apoptotic, calcium/calmodulin-dependent serine/threonine kinase. Here, we report that DAP kinase phosphorylates the regulatory light chain of myosin II (MLC) both in vitro and in vivo, and that this phosphorylation occurs preferentially at residue Ser19. In quiescent fibroblasts, DAP kinase stabilizes stress fibers through phosphorylation of MLC, but it is dispensable for the formation of peripheral microfilament bundles. This cytoskeletal effect of DAP kinase occurs before the onset of apoptosis and does not require an intact death domain. In addition, DAP kinase is required for serum-induced stress-fiber formation, which is associated with the upregulation of its catalytic activity. Despite being both sufficient and necessary for the assembly or maintenance of stress fibers, DAP kinase is incapable of stimulating the formation of focal adhesions in quiescent cells. Moreover, it promotes the disassembly of focal adhesions but not stress fibers in cells receiving serum factors. Together, our results identify a novel and unique function of DAP kinase in the uncoupling of stress fibers and focal adhesions. Such uncoupling would lead to a perturbation of the balance between contractile and adhesion forces and subsequent cell detachment, which might contribute to its pro-apoptotic activity.

Key words: DAP kinase, Myosin phosphorylation, Stress fibers, Focal adhesions, Apoptosis

Introduction

The regulation of actomyosin contractility plays a key role in various cellular functions, such as smooth-muscle contraction (Tan et al., 1992; Fukata et al., 2001), stress fiber and focal adhesion formation (Tan et al., 1992; Chrzanowska-Wodnicka and Burridge, 1996), neuronal morphogenesis (Lin et al., 1996; Amano et al., 1998; Wylie et al., 1998), cytokinesis (De Lozanne and Spudich, 1987; Komatsu et al., 2000) and apoptotic membrane blebbing (Mills et al., 1998). In both smooth muscle and nonmuscle cells, actomyosin contractility is primarily regulated by phosphorylation of the regulatory light chain of myosin II (MLC) (Tan et al., 1992). Phosphorylation of MLC at Thr18 and Ser19 enhances the actin-activated myosin motor activity (Kamm and Stull, 1989), whereas phosphorylation at Ser1/2 and Thr9 by protein kinase C (PKC) decreases its affinity for actin (Bengur et al., 1987). Phosphorylation of MLC at Thr18 and Ser19 is regulated by the MLC kinases and phosphatases. Myosin-light-chain kinase (MLCK) is responsible for calcium-dependent MLC phosphorylation at the stimulatory sites (Kamm and Stull, 1989). Rho kinase (ROCK) can both inactivate MLC phosphatase by phosphorylating its myosin-binding subunit (Kimura et al., 1996; Feng et al., 1999; Kawano et al., 1999) and directly phosphorylate MLC at MLCK site (Amano et al., 1996; Ueda et al., 2002). The p21-activated kinase was also reported to increase MLC phosphorylation in certain cell types (Kiosses et al., 1999; Sells et al., 1999). Through alterations of the MLC phosphorylation, these kinases and phosphatases regulate actomyosin contractile forces, profoundly influencing cell motility and morphology (Wilson et al., 1991; Klemke et al., 1997; Dong et al., 1998; Fukata et al., 1999).

Contractility of nonmuscle cells is intimately associated with adhesion and adhesion-dependent signaling. Focal adhesions are sites of contact between the cell surface and the extracellular matrix, where the associated stress fibers terminate. They consist of both structural proteins that link integrins to the actin cytoskeleton and signaling proteins that transduce a range of adhesion-dependent signals (Sastry and Burridge, 2000; Zamir and Geiger, 2001). The assembly and stabilization of focal adhesions is greatly influenced by myosin-II-driven contractility. Chemicals that inhibit either MLC phosphorylation or actin-dependent myosin ATPase activity cause a rapid loss of both stress fibers and focal adhesions (Volberg et al., 1994; Chrzanowska-Wodnicka and Burridge, 1996). Similar effects are observed by dominant-negative inhibition of myosin IIA heavy chain (Wei and Adelstein, 2000) or overexpression of caldesmon (Helfman et al., 1999), a protein that restrains the interactions of myosin heads with actin filaments. Conversely, activation of the Rho/ROCK pathway stimulates the formation of both stress fibers and their associated focal adhesions in a contractility-dependent manner (Amano et al., 1997; Uehata et al., 1997).

MLC phosphorylation is also involved in apoptosis. Death-associated protein kinase (DAP kinase) is a

calcium/calmodulin-dependent serine/threonine kinase and was cloned based on its involvement in interferon-γ-induced apoptosis in HeLa cells (Deiss et al., 1995). Subsequently, this kinase was demonstrated to participate in various apoptotic systems (Cohen et al., 1997; Cohen et al., 1999; Inbal et al., 1997; Pelled et al., 2001; Raveh et al., 2001; Jang et al., 2002; Yamamoto et al., 2002). Furthermore, ectopic expression of DAP kinase in several cell lines promotes apoptosis (Cohen et al., 1997) but the mechanisms of this have not been fully understood. Because its catalytic activity is required for the death-promoting function (Cohen et al., 1997), DAP kinase is likely to function by phosphorylating certain intracellular substrates. DAP kinase phosphorylates MLC in vitro (Cohen et al., 1997) but its in vivo substrates remain to be identified. DAP kinase was also found to activate $p19^{ARF}$ (Raveh et al., 2001) and a random peptide-based analysis for determining the preferred sites of DAP-kinase phosphorylation predicts $p19^{ARF}$ to be a potential substrate of DAP kinase (Velentza et al., 2001). ZIP kinase, whose kinase domain is closely related to DAP kinase, was shown to phosphorylate MLC in both smooth muscle (Murata-Hori et al., 1999; Niiro and Ikebe, 2001) and nonmuscle cells (Murata-Hori et al., 2001). Furthermore, the mouse homolog of DAP kinase (mDAP kinase) can phosphorylate MLC at both Thr18 and Ser19 in vitro, and can increase the MLC phosphorylation levels in cells treated with tumor necrosis factor α (TNF-α) (Jin et al., 2001). Whether mDAP kinase itself induces MLC phosphorylation in vivo is, however, currently unknown. DAP kinase is localized to the actin cytoskeleton and this localization is important for its pro-apoptotic function (Cohen et al., 1997). However, whether DAP kinase affects cytoskeletal organization and dynamics, and whether such a cytoskeletal effect plays any role in its pro-apoptotic activity have yet to be determined.

Although DAP kinase functions as a positive mediator of apoptosis induced by various extracellular and intracellular factors (Deiss et al., 1995; Cohen et al., 1997; Cohen et al., 1999; Inbal et al., 1997; Pelled et al., 2001; Raveh et al., 2001; Jang et al., 2002; Yamamoto et al., 2002), little is known about how DAP kinase is regulated by these stimuli. Transcriptional regulation of DAP kinase is known to occur in transforming growth factor β (TGF-β)-induced, DAP-kinase-mediated apoptosis (Jang et al., 2002). Post-translationally, DAP kinase can undergo an inhibitory autophosphorylation at a serine residue in its calmodulin-binding domain, and this is reversed during C6-ceramide-induced apoptosis (Shohat et al., 2001). Thus, DAP kinase activity is not only regulated by intracellular calcium levels; other signaling pathways are likely to influence its activity and function.

In this study, we examined the cytoskeletal effects of DAP kinase. We found DAP kinase phosphorylates MLC at Ser19 in vitro and increases the level of phosphorylated MLC in vivo. In fibroblasts, DAP kinase stabilizes stress fibers in a contractility-dependent manner and mediates serum-induced stress-fiber formation. However, unlike other proteins and factors that induce both MLC phosphorylation and stress-fiber formation (Ridley and Hall, 1992; Leung et al., 1996; Amano et al., 1997; Totsukawa et al., 2000; Katoh et al., 2001), DAP kinase is insufficient to promote the assembly of focal adhesions in serum-starved fibroblasts, and can even induce focal adhesion disassembly without affecting stress fibers in cells cultured in serum-containing medium. These findings not only reveal a novel function of DAP kinase in the regulation of actin cytoskeleton but also imply a role for DAP kinase in perturbing the balance between cell contractile and adhesion forces, which might contribute to its apoptosis-promoting activity.

Materials and Methods

Plasmids

DAPKΔCaM and DAPKΔDD (Cohen et al., 1997) were constructed using the Quick-Change site-directed mutagenesis kit (Stratagene). Human MLC cDNA was cloned by reverse-transcription polymerase chain reaction (RT-PCR) using RNA extracted from Hep3B cells, and various MLC mutants were generated by site-directed mutagenesis. These MLC cDNAs were cloned into pGEX-4T vector to generate glutathione-*S*-transferase (GST) fusion proteins or into the pRK5 vector for expression in mammalian cells. Production of the GST fusion proteins and GST pull-down analysis were performed as described (Tsai et al., 2000).

Antibodies and reagents

The polyclonal antibody to DAP kinase has been described previously (Jang et al., 2002). Two antibodies to diphosphorylated MLC were used in this study; one has been described previously (Ratcliffe et al., 1999) and another was from Santa Cruz Biotechnology. An antibody specific to MLC phosphorylated at Ser19 (P1) has been described (Murata-Hori et al., 2001). Antibodies to MLC, MLCK and the Flag epitope were obtained from Sigma. Antibodies to FAK and paxillin were from Transduction Laboratories; antibodies to vinculin and β1-integrin were from Chemicon. Y27632, 2,3-butanedione 2-monoxime (BDM) and 1-(5-iodonaphthalene-1-sulfonyl)homopiperazine (ML-7) were from Calbiochem. Cytochalasin D was from Sigma.

Cell culture, transfection and retroviral infection

293T and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Transfection of NIH3T3 cells was performed using the Lipofectamine-Plus reagent (Life Technologies). 293 and 293T cells were transfected using the calcium phosphate method. Generation of recombinant retroviruses and infection of NIH3T3 cells were carried out following procedures described previously (Tsai et al., 2000).

Production of baculovirus

Monolayers of Sf-21 cells cultured in Grace's medium (Life Technologies) supplemented with 10% FCS were co-transfected with linearized virus DNA (BaculoGold, Pharmingen) and the pVL1392 vector containing the DAP-kinase cDNA. The recombinant virus was harvested, amplified and then used to infect monolayers of Sf-21 cells in TMN-FH medium (Applichem). After a 3-day incubation, the Flag-tagged DAP kinase was purified using the anti-Flag M2 agarose (Sigma).

Immunoprecipitations and in vitro kinase assays

Cells were lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS] supplemented with 1 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM sodium vanadate, 4 mM sodium pyrophosphate and 20 mM NaF. The lysate was clarified by centrifugation and DAP kinase, ROCK or MLCK was immunoprecipitated using anti-Flag, anti-Myc or anti-MLCK antibody, respectively, followed by protein-A/Sepharose

conjugated to rabbit anti-mouse antibody as described previously (Tsai et al., 2000). The immune complex recovered was washed with lysis buffer and then with kinase buffer containing 50 mM HEPES (pH 7.5), 8 mM $MgCl_2$ and 2 mM $MnCl_2$. Kinase reaction was carried out in kinase buffer supplemented with 0.5 mM $CaCl_2$, 50 μM ATP, 10 μCi [γ-^{32}P]ATP, 0.1 mg ml^{-1} bovine serum albumin (BSA), 1 μM bovine calmodulin (Sigma), 3 μg bovine muscle MLC (Sigma) and/or recombinant GST-MLC at 25°C for 15 minutes.

Preparation of detergent-soluble and insoluble fractions

Cells were extracted with 0.5 ml of MES buffer (50 mM MES, pH 6.8, 2.5 mM EDTA and 2.5 mM $MgCl_2$) containing 0.5% Triton X-100 for 3 minutes. The supernatant (soluble fraction) was collected by centrifugation at 16,000 ***g*** for 2 minutes, precipitated by ethanol and then resuspended in 200 μl RIPA buffer. The detergent-insoluble matrix was extracted with 200 μl RIPA buffer.

Phosphoamino acid analysis

Bovine muscle MLC phosphorylated by DAP kinase at 25°C for 20 minutes was excised from the PVDF membrane and then hydrolysed using HCl. Amino acids and the phosphoamino acid standards were separated by two-dimensional thin-layer electrophoresis as described (Chen et al., 1995).

Immunofluorescence

Cells were fixed with 3.7% paraformaldehyde for 20 minutes and then permeabilized with extraction buffer containing 50 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM $MgCl_2$ and 0.5% Triton X-100 for 5 minutes. Cells were blocked with PBS supplemented with 10% goat serum, 1% BSA and 50 mM NH_4Cl for 1 hour, and then incubated with various primary antibodies diluted in PBS containing 0.2% BSA and 5% goat serum for 1 hour. Cells were then incubated with FITC, rhodamine or AMCA (7-amino-4-methylcoumarin-3-acetic acid)-conjugated anti-mouse or anti-rabbit secondary antibody for 1 hour. Alternatively, cells were incubated with 0.1 μM rhodamine-conjugated phalloidin (Molecular Probes) for 1 hour. Cells were then washed, mounted and examined with a Nikon 024632 or Leica DM RA epifluorescence microscope, using a 40× or 100× objective lens. Fluorescent images were photographed using Kodak Elite Chrome 400 film and digitalized with a slide scanner (Nikon).

Reduction of endogenous DAP-kinase expression using siRNA

A 19-nucleotide small interfering RNA (siRNA) duplex with 3′ TT overhangs corresponding to the DAP kinase mRNA (CACCAGUACCCUUGCCAAA) was synthesized and labeled by FITC (Orbigen). siRNA transfections were performed using the RNA interference (RNAi) Shuttle™ Transfection Kit (Orbigen) according to the manufacturer's protocol. To achieve a high efficiency of transfection, cells were transfected two or three times at 24-hour intervals. After transfection, cells were harvested for western-blot analysis or plated onto cover slides for serum stimulation followed by immunofluorescence analysis.

Interference reflection microscopy

Interference reflection microscopy (IRM) was carried out using a Leica TCS SP2 confocal laser scanning microscope set in the reflection mode and equipped with a 63× oil objective lens. A selective interference excitation filter (488 nm) was used. The IRM image corresponding to the basal surface of the cells was taken by the MetaMorph Imaging System and then processed with the Adobe Photoshop software.

Results

DAP kinase phosphorylates MLC in vitro and in vivo

DAP kinase was shown to phosphorylate MLC in vitro and its kinase domain is distantly related to that of MLCK (Cohen et al., 1997; Inbal et al., 2000). We first investigated whether there is a direct interaction between these two proteins. GST pull-down analysis demonstrated that recombinant DAP kinase purified from baculovirus could physically interact with the GST-MLC fusion protein but not with the GST alone (Fig. 1A). Furthermore, when immunoprecipitated DAP kinase was used to phosphorylate proteins in cell lysates, a 20 kDa protein that was present only in the Triton X-100-insoluble fraction (not in the soluble fraction) was revealed as the most prominent protein phosphorylated by DAP kinase (Fig. 1B). This protein co-migrated with recombinant MLC, suggesting that MLC is a major substrate of DAP kinase. To determine the sites of MLC phosphorylation, we generated GST-MLC proteins in which the Ser1/2 or Thr18/Ser19 of MLC were mutated to Ala. The wild-type and Ser1/2 mutant of GST-MLC were phosphorylated equally well by DAP kinase, whereas the Thr18/Ser19 mutant was not phosphorylated (Fig. 1C). Furthermore, the kinase-dead mutant, DAPK42A, did not result in MLC phosphorylation. These results indicate that DAP kinase phosphorylates MLC at the MLCK sites (Ser19 and/or Thr18) instead of the PKC sites (Ser1/2). To determine whether DAP kinase preferentially phosphorylates Ser19 or Thr18 of MLC, phosphoamino-acid analysis on the wild-type MLC phosphorylated by DAP kinase was performed to reveal that MLC was phosphorylated exclusively on Ser (Fig. 1D), indicating that DAP kinase is an MLC Ser19 kinase.

To test whether DAP kinase phosphorylates MLC in vivo, NIH3T3 cells were transiently transfected with expression vector for wild-type DAP kinase or DAPK42A, and MLC phosphorylation status was assessed by an antibody specifically recognizing phosphorylated MLC (Ratcliffe et al., 1999). Because this antibody reacts only with the diphosphorylated MLC (i.e., phosphorylated at both Thr18 and Ser19), it is possible that cells contain a certain level of MLC that is phosphorylated only at Thr18, so that overexpression of DAP kinase would increase the overall cellular level of the diphosphorylated MLC. Alternatively, and more probably, MLC phosphorylated at Ser19 by DAPK might become diphosphorylated by other MLC kinases, such as ROCK and ZIP kinase. Indeed, ectopic expression of DAP kinase in NIH3T3 cells enhanced MLC phosphorylation, whereas expression of DAPK42A, previously shown to function as a dominant-negative mutant (Cohen et al., 1999), resulted in a marked reduction in MLC phosphorylation in vivo (Fig. 1E). Because DAPK42A did not nonspecifically inhibit other MLC kinases, such as ROCK and MLCK (see below), our result indicates that DAP kinase is capable of phosphorylating MLC in vivo. A similar induction of phosphorylated MLC was observed from 293 cells transfected with DAP kinase (Fig. 1E). Furthermore, the ability of ectopically expressed DAP kinase to promote MLC phosphorylation in vivo was also confirmed by a commercially available antibody to phosphorylated MLC (data not shown) and by antibody P1, which specifically recognizes MLC phosphorylated at Ser19 (Murata-Hori et al., 2001) (Fig. 1E). Together, these results provide strong evidence that DAP kinase can function as an MLC kinase in vivo.

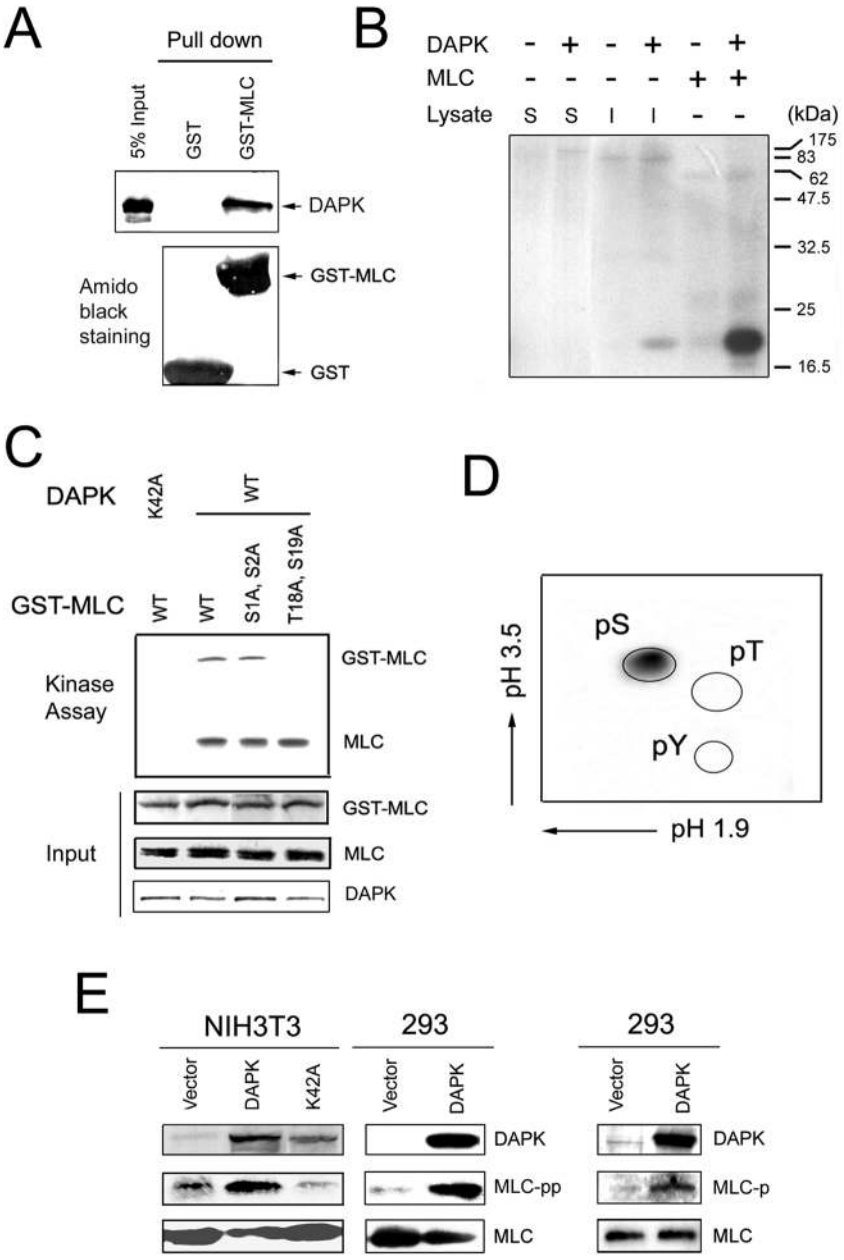


Fig. 1. DAP-kinase phosphorylates MLC in vitro and in vivo. (A) DAP-kinase interacts with MLC. GST-MLC fusion protein or GST alone immobilized on glutathione-Sepharose beads was incubated with DAP kinase purified from recombinant baculovirus. The precipitates and the purified DAP kinase (5% of the input amount) were subjected to SDS-PAGE and immunoblotted with anti-DAP-kinase antibody (top). The same membrane was stained by amido black to show equal input of the GST and GST-MLC proteins (bottom). (B) DAP kinase phosphorylates a ~20 kDa protein. The Triton-soluble (S) and -insoluble (I) fractions of the 293 cell lysate were separated, heated to inactivate cellular kinases and then used as substrates in the kinase reactions catalysed by Flag/DAP-kinase immunoprecipitated from lysates of transfected cells. The anti-Flag immunoprecipitates derived from untransfected cells (DAPK–) and purified MLC were included as negative and positive controls, respectively. (C) Determination of MLC phosphorylation sites by DAP kinase. Wild-type and mutant MLC fused with GST were phosphorylated in vitro by Flag/DAP-kinase or its kinase-dead mutant (K42A) immunoprecipitated from transfected cells. Wild-type MLC (MLC) was added to each kinase reaction as an internal control. The kinase reactions were subjected to SDS-PAGE and transferred to PVDF membrane. The membrane was stained by amido black to reveal the equal input of MLC, GST-MLC and DAP kinase in the kinase reactions, and then subjected to autoradiography to detect the phosphorylated proteins. (D) Phosphoamino-acid analysis of MLC phosphorylated by Flag-DAP kinase. The positions of phosphorylated serine (pS), threonine (pT) and tyrosine (pY) are indicated. (E) DAP kinase enhances MLC phosphorylation in vivo. NIH3T3 cells infected with recombinant retrovirus as indicated or 293 cells transfected with various constructs were serum-starved for 6 hours. Cell lysates were then subjected to immunoblot with antibodies specific to DAP kinase, diphosphorylated MLC (MLC-pp), monophosphorylated MLC (MLC-p) or MLC as indicated.

DAP kinase stabilizes stress fibers in quiescent cells

Phosphorylation of MLC at Ser19 is known to increase the actomyosin contractility, which are associated with the formation of stress fibers (Chihara et al., 1997; Kawano et al., 1999). To investigate whether DAP kinase plays a positive role in the assembly of stress fibers, NIH3T3 cells were infected with retrovirus carrying DAP kinase, DAPK42A or DAPKΔCaM (a mutant with constitutive kinase activity) (Cohen et al., 1997), together with the puromycin-resistance gene. After selection with puromycin for 2 days, pools of infected cells were generated that overexpress the DAP kinase or its mutants (Fig. 2A). The expression level of DAPK in the overexpressing cells was about nine times higher than its endogenous level. Because prolonged culture of the cells carrying DAP kinase or DAPKΔCaM virus resulted in apoptosis, populations of cells immediately after selection were serum starved and then used for examination of their actin cytoskeletons. In cells infected with the control virus, serum starvation for 6 hours caused a drastic loss of stress fibers, whereas cells infected with DAP-kinase virus contained many stress fibers (Fig. 2B). In cells expressing DAPKΔCaM, not only was the proportion of cells containing stress fibers increased slightly (Fig. 2C) but also thicker stress fibers were found (Fig. 2B). In cells treated with BDM, an inhibitor of myosin ATPase activity and actin-myosin interaction (Higuchi and Takemori, 1989), maintenance of stress fibers induced by DAP kinase or DAPKΔCaM was totally abolished (Fig. 2B), suggesting that the actomyosin contractility is essential for this effect of DAP kinase. Interestingly, in cells expressing DAPK42A thick actin bundles were still evident at the cell periphery but stress-fiber formation was not observed in the cell body (Fig. 2B), suggesting that the activity of DAP kinase

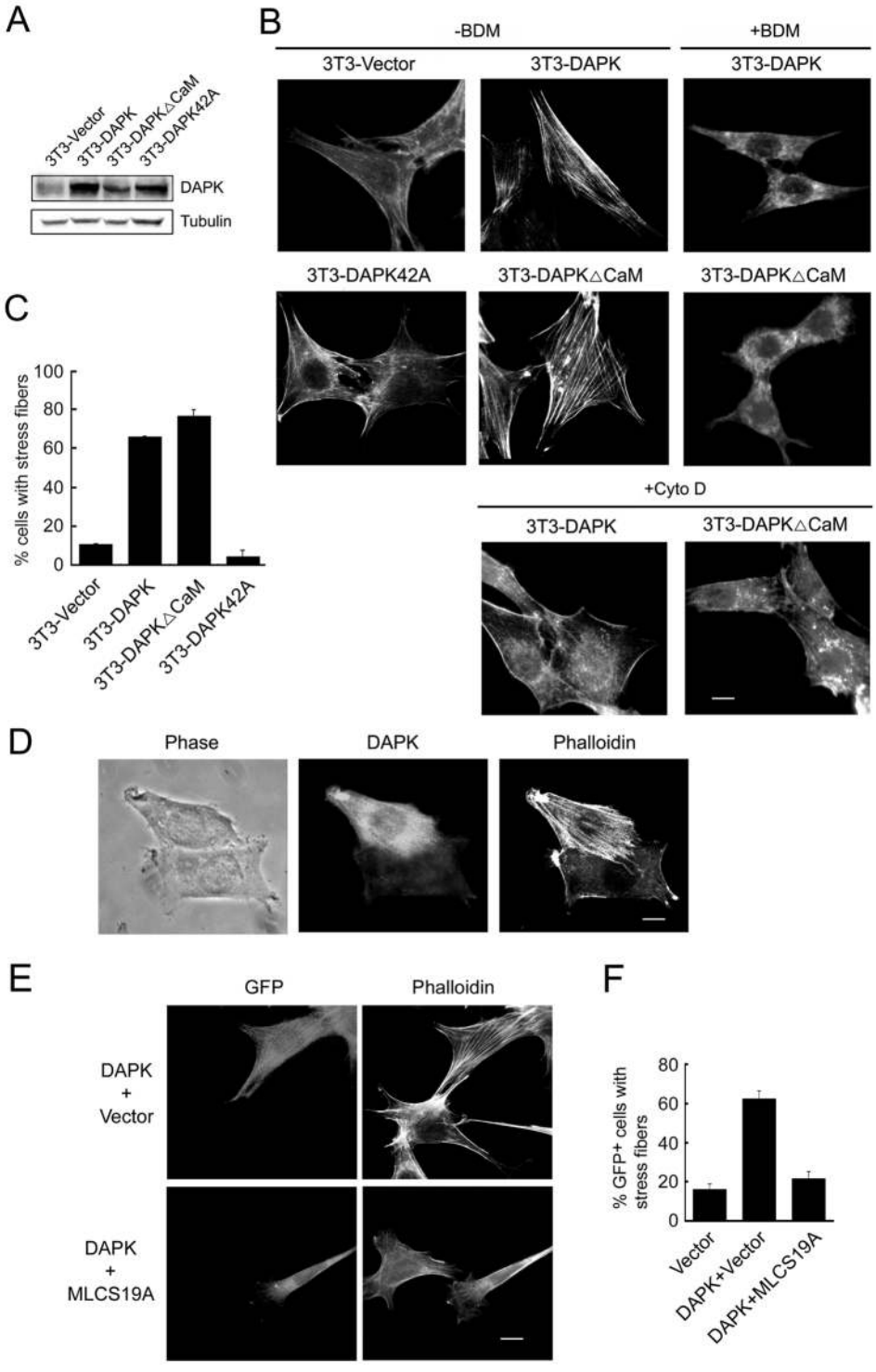


Fig. 2. DAP kinase promotes the assembly or stabilization of stress fibers. (A) NIH3T3 cells were infected with recombinant retrovirus carrying vector alone or various forms of DAP kinase as indicated. Infected cells were selected by puromycin and then subjected to immunoblot analysis to detect the expression of DAP kinase proteins. (B) Cells as in (A) were serum-starved for 6 hours, treated with or without 25 mM BDM for 10 minutes or 100 nM cytochalasin D for 30 minutes, and then stained with rhodamine-phalloidin. (C) Quantitation of cells with stress fibers in experiments as described in (B). Only cells that were not exposed to BDM and cytochalasin D were analysed. The values shown are means ± s.d. from at least three independent experiments and more than 300 individual cells were counted for each experiment. (D) NIH3T3 cells transiently transfected with DAP kinase were serum starved and then double stained with anti-DAP-kinase and rhodamine-phalloidin. A cell that overexpresses DAP kinase is indicated by strong DAP kinase staining pattern. (E) NIH3T3 cells were co-transfected with DAP kinase, MLCS19A mutant (or a control vector) and GFP at a ratio of 5:5:1. Cells were serum starved and stained with rhodamine-phalloidin. (F) Quantitation of GFP-positive cells with stress fibers in experiments described in (E). Scale bars, 10 μm.

is dispensable for the formation of cortical actin bundles. To confirm the effect of DAP kinase on F-actin, a transient transfection system was used. Similarly, overexpression of DAP kinase in NIH3T3 cells (Fig. 2D) or HeLa cells (data not shown) resulted in the maintenance of stress fibers under serum starvation. This DAP-kinase-induced stabilization of stress fibers was abrogated by co-transfection with an MLC mutant in which Ser19 is mutated into Ala (MLCS19A) (Fig. 2E,F), suggesting that the cytoskeletal reorganization is primarily mediated by phosphorylation of MLC. Finally, to substantiate the identity of the fibrous structures observed in cells overexpressing DAP kinase or DAPKΔCaM, these cells were treated with cytochalasin D, an inhibitor of actin polymerization. Cytochalasin-D treatment caused a drastic reduction of stress fibers in cells infected with DAP kinase or DAPKΔCaM virus (Fig. 2B). Thus, the morphology of these fibrous structures, together with their sensitivity to both BDM and cytochalasin D, unequivocally identifies them as stress fibers. In summary, these results demonstrate that DAP kinase can stabilize stress fibers in quiescent cells, probably because of effects on MLC phosphorylation and actomyosin contractility.

Effect of DAP kinase on F-actin does not require its death domain and is not a consequence of apoptosis

The death domain of DAP kinase plays a positive role in its ability to induce apoptosis (Cohen et al., 1999). To determine whether this domain is required for its cytoskeletal effects, we

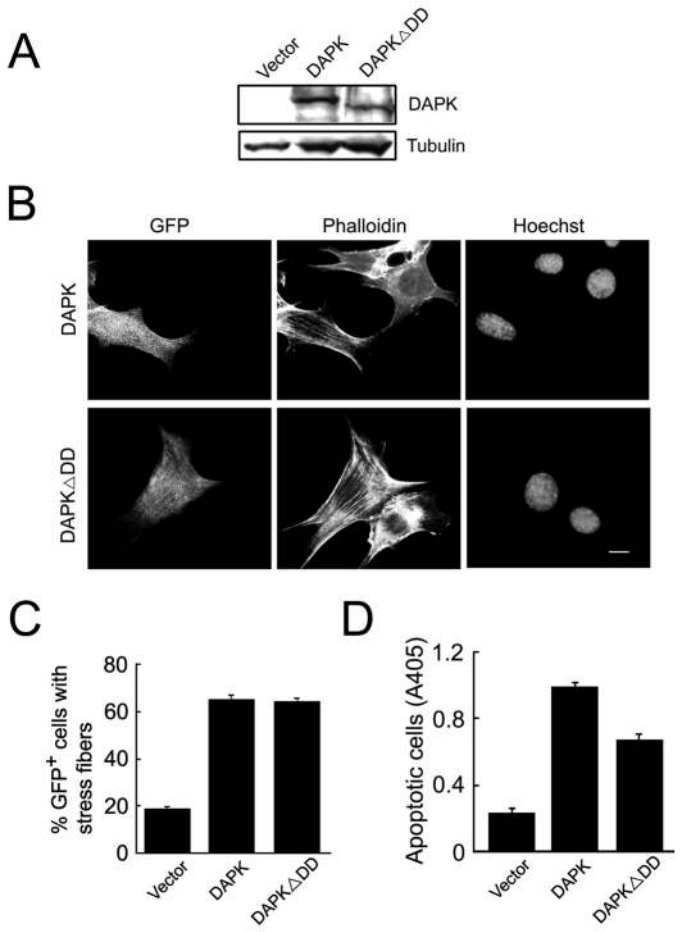


Fig. 3. Maintenance of stress fibers by DAP kinase does not require its death domain. (A) NIH3T3 cells were co-transfected with vector for wild-type DAP kinase or DAPKΔDD and the GFP expression vector at a ratio of 10:1. Cells were serum starved for 6 hours, lysed and subjected to western blot analysis. (B) Alternatively, serum-starved transfectants were double stained with rhodamine-phalloidin and Hoechst 33258. Transfected cells are visualized by GFP fluorescence. Scale bar, 10 μm. (C) Quantitation of GFP-positive cells with stress fibers in experiments as described in (B). (D) NIH3T3 cells were transiently transfected with control vector, DAP kinase or DAPKΔDD. Two days after transfection, cells were re-fed with serum-free medium and then cultured for another 18 hours. Apoptotic cells were determined by cell death detection ELISA as described previously (Wang et al., 2002).

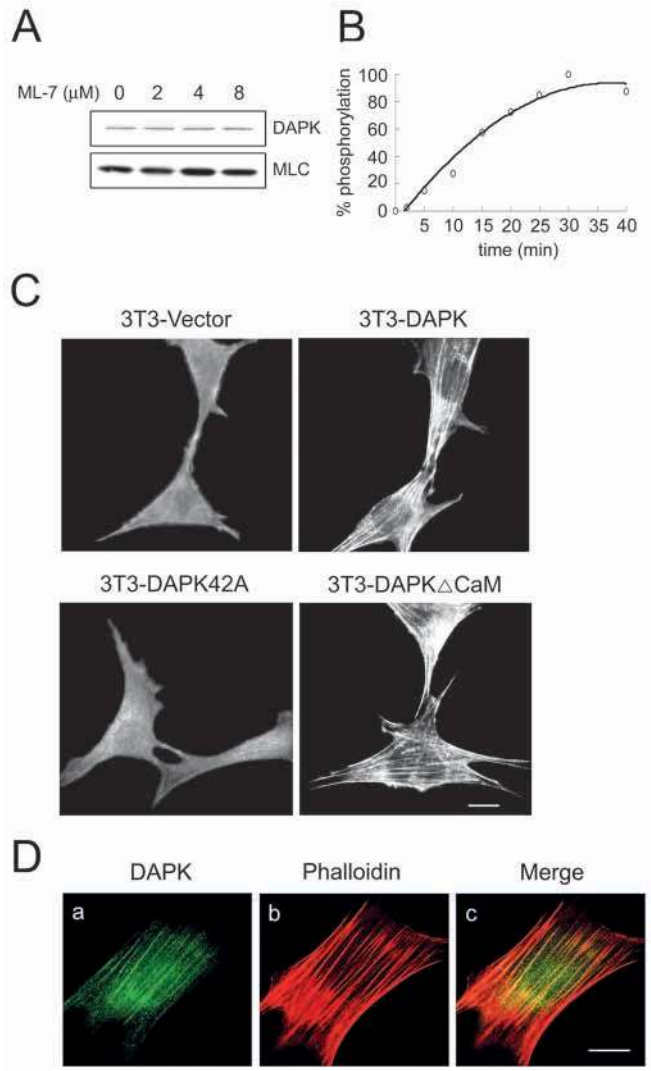


Fig. 4. DAP kinase and MLCK display distinct roles in the assembly of stress fibers. (A) DAP kinase is insensitive to ML-7. ML-7 was added at indicated concentrations to kinase reactions containing MLC, Flag-DAP kinase and Ca^{2+}/calmodulin. MLC phosphorylation (bottom) and DAP kinase autophosphorylation (top) were detected by autoradiography. (B) Kinase reactions as in (A) but without ML-7 were performed for various times. The extent of phosphorylation was quantified and expressed as percentage, assigning the maximum phosphorylation of MLC to 100%. (C) Virus-infected NIH3T3 cells (Fig. 2B) were serum starved and treated with 5 μM of ML-7 for 30 minutes. Cells were then fixed and stained with rhodamine-phalloidin. (D) Subcellular localization of endogenous DAP kinase. NIH3T3 cells cultured in serum-containing medium were double stained with anti-DAP-kinase for endogenous DAP kinase (a) and with rhodamine-phalloidin for F-actin (b). The merged image is shown in (c). Scale bars, 10 μm.

generated a deletion mutant (DAPKΔDD) that lacks the entire death domain and a short C-terminal tail. This mutant or the wild-type DAP kinase was transfected into NIH3T3 cells together with an expression plasmid for green fluorescent protein (GFP). After serum starvation for 6 hours, cells expressing DAPKΔDD (as visualized by GFP fluorescence) displayed stress fibers indistinguishable from those expressing the wild-type DAP kinase (Fig. 3B). Furthermore, the proportion of DAPKΔDD-expressing cells that contained stress fibers was similar to that of cells expressing wild-type DAP kinase (Fig. 3C). Western-blot analysis revealed the expression of this DAPKΔDD mutant, which showed a faster electrophoretic mobility than the wild-type protein (Fig. 3A). Cells expressing either the wild-type DAP kinase or DAPKΔDD did not exhibit apoptosis-like nuclear morphology [i.e. nuclear condensation and fragmentation (Fig. 3B) or DNA fragmentation (data not shown)] by the time they were assayed for the formation of stress fibers. Nevertheless, apoptosis did occur later, 18 hours after serum starvation (Fig. 3D). Consistent with previous work (Cohen and Kimchi, 2001), the deletion of the death domain reduced, but did not completely

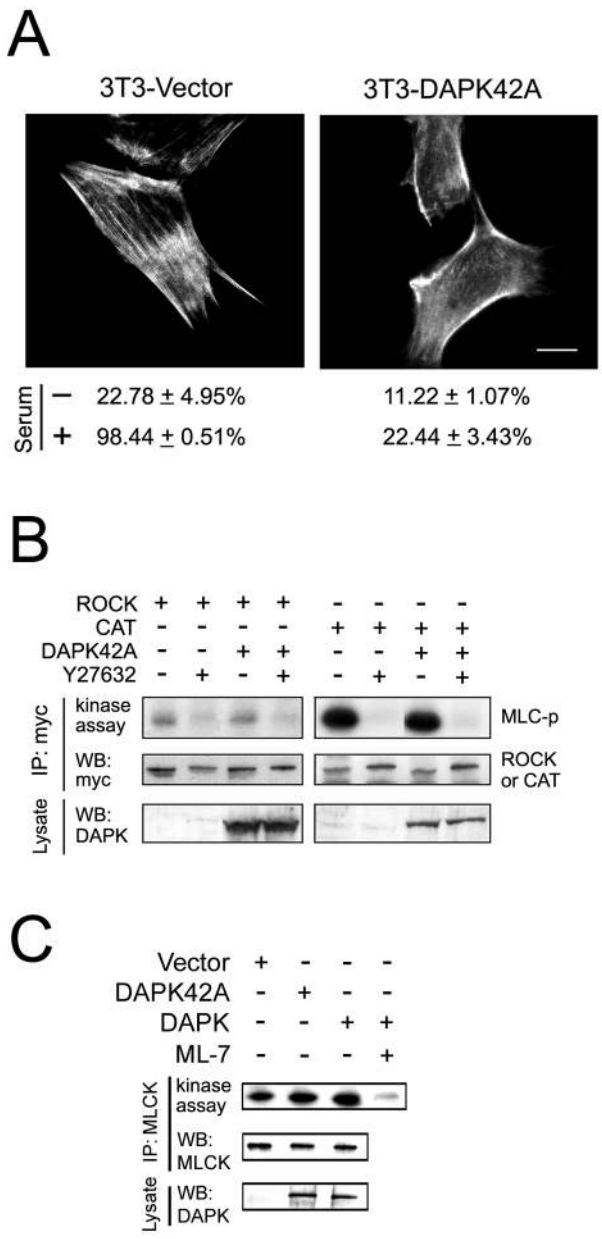


Fig. 5. DAP kinase is involved in serum-induced stress-fiber formation. (A) NIH3T3 cells were infected with recombinant retrovirus carrying vector alone or DAPK42A, as indicated. Serum-starved cells were re-stimulated with serum for 20 minutes and then stained with rhodamine-phalloidin. Scale bar, 10 μm. The percentages of cells with stress fibers seen in serum-starved and stimulated conditions are indicated on the bottom. (B) NIH3T3 cells were transiently transfected with various combinations of constructs as indicated. Myc-ROCK and Myc-CAT were immunoprecipitated from cell lysates and then used to phosphorylate recombinant MLC in vitro in the presence or absence of 2 μM Y27632. The kinase reactions were resolved by SDS-PAGE and MLC phosphorylation was detected by autoradiography (top). The same kinase reactions were subjected to immunoblot analysis to detect the precipitated ROCK or CAT (middle). The expression of DAPK42A was detected by immunoblot (bottom). (C) Endogenous MLCK was immunoprecipitated from lysates of 293T cells transfected with various constructs, as indicated. The immunoprecipitates were used to phosphorylate recombinant MLC in vitro, in the presence or absence of 2 μM ML-7 (top). The same kinase reactions were subjected to immunoblot analysis to detect equal input of MLCK (middle). The expression of various DAP-kinase proteins was detected by immunoblot (bottom).

eliminate, its apoptotic inducibility. Our results indicate that the effect of DAP kinase on stress fiber maintenance requires kinase activity but not an intact death domain. Furthermore, the observed cytoskeletal effects occur before the onset of apoptosis and thus seem unlikely to be a consequence of the DAP-kinase-induced apoptosis.

DAP kinase and MLCK play distinct roles in the assembly of actin filaments

DAP kinase and MLCK can both phosphorylate MLC at Ser19, so we investigated whether these two enzymes possess any distinct biological functions. If they had different sensitivities to certain chemical inhibitors, this could provide useful reagents for distinguishing the cellular functions of the two kinases. ML-7, a selective inhibitor of MLCK, was found to be incapable of inhibiting the activity of ZIP kinase (Niiro and Ikebe, 2001), a DAP-kinase-family protein. Likewise, DAP-kinase activity was not affected by addition of ML-7 to the kinase reaction, even though the concentration of ML-7 used was as high as 8 μM (Fig. 4A), 25-fold higher than its K_i for MLCK (Saitoh et al., 1987). Kinetic analysis indicated that the time course used for assaying DAP kinase activity (15 minutes) was within the linear range of the kinase reaction (Fig. 4B), and should therefore reveal any inhibitory effects of the compound. Consistent with the insensitivity of DAP kinase to ML-7, this inhibitor did not block the stress fiber formation seen in DAP-kinase- or DAPKΔCaM-overexpressing cells. However, in accordance with previous studies (Totsukawa et al., 2000; Katoh et al., 2001), ML-7 greatly reduced the assembly of cortical actin bundles, which is most evident by comparing DAPK42A cells receiving ML-7 (Fig. 4C) with those without ML-7 (Fig. 2B). The ability of ML-7, but not DAPK42A, to inhibit the formation of peripheral microfilament bundles not only indicates an involvement of MLCK, but not DAP kinase, in the assembly of these filaments but also confirms that the effect of DAPK42A is specific for inhibiting endogenous DAP kinase without affecting other related kinases, such as MLCK. Thus, DAP kinase and MLCK appear to play distinct roles in the spatial assembly of actin filaments.

The role of MLCK in the assembly of peripheral microfilament bundles is due, at least in part, to its localization to cell periphery (Totsukawa et al., 2000). To investigate whether the role of DAP kinase in the assembly of central actin filaments is correlated with its intracellular localizations, we examined the distribution of endogenous DAP kinase. When NIH3T3 cells grown in serum-containing medium were double stained with anti-DAP-kinase and phalloidin, we observed that a significant proportion of endogenous DAP kinase was distributed with stress fibers (Fig. 4D), consistent with its previously reported localization to the actin cytoskeleton (Cohen et al., 1997). However, DAP kinase was not evenly distributed on stress fibers but was concentrated in the central portion of cells and virtually undetectable at cell periphery.

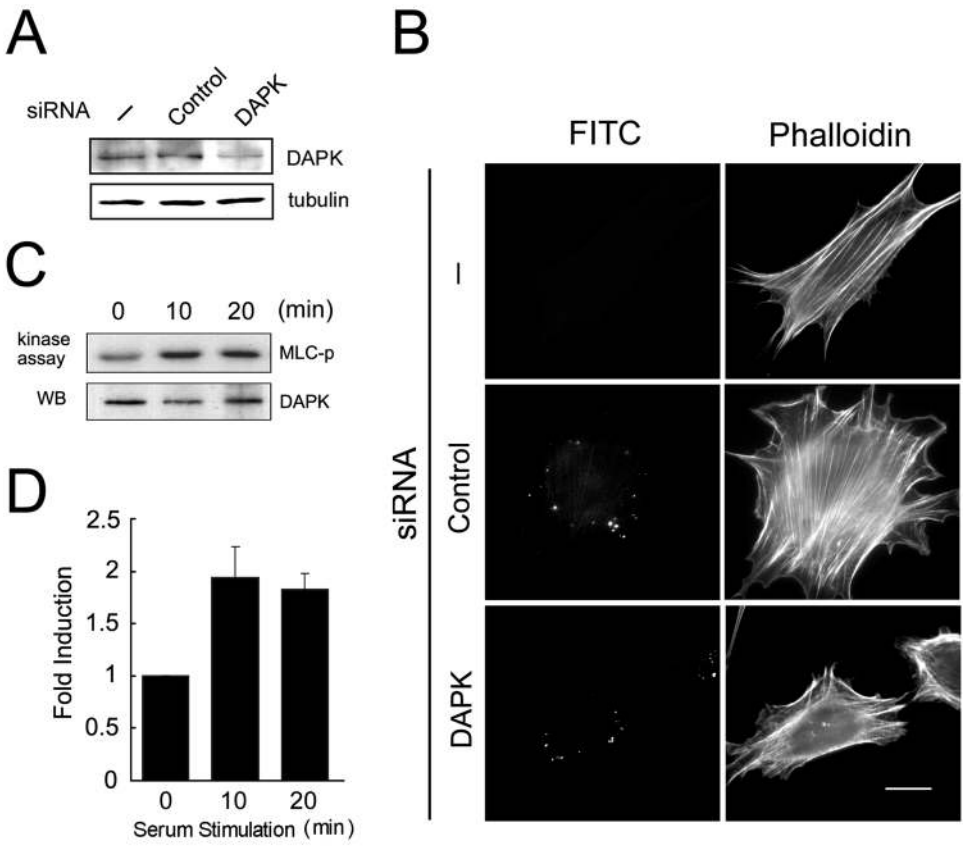


Fig. 6. DAP kinase is required for serum-induced stress fiber formation and is activated by serum. (A) Reduction of endogenous DAP kinase expression by siRNA. Cell lysates from untreated (–), control-siRNA- or DAPK-siRNA-treated cells were immnuoblotted with antibodies as indicated. (B) siRNA-mediated knockdown of DAP kinase inhibits stress-fiber formation in response to serum. Cells as in (A) were stimulated with serum for 20 minutes and then stained with rhodamine-phalloidin. Cells receiving siRNA were visualized by their FITC fluorescence. Scale bar, 10 μm. (C) NIH3T3 cells were transfected with Flag-DAP kinase and stimulated with or without serum for various times. The Flag-DAP kinase was immunoprecipitated from cell lysates and subjected to in vitro kinase reactions using MLC as a substrate. The kinase reactions were resolved by SDS-PAGE, and MLC phosphorylation was detected by autoradiography (top). The same kinase reactions were subjected to immunoblot analysis to detect the precipitated Flag-DAP kinase (bottom). The result shown is a representative experiment from three independent experiments. (D) Quantification of DAP kinase activities in response to serum stimulation. Experiments were performed as in (C), and the amounts of phosphorylated MLC were normalized by those of DAP kinase input in the kinase reactions. Data are expressed as fold inductions relative to cells that were not stimulated by serum. Each value represents means ± s.d. from three independent experiments.

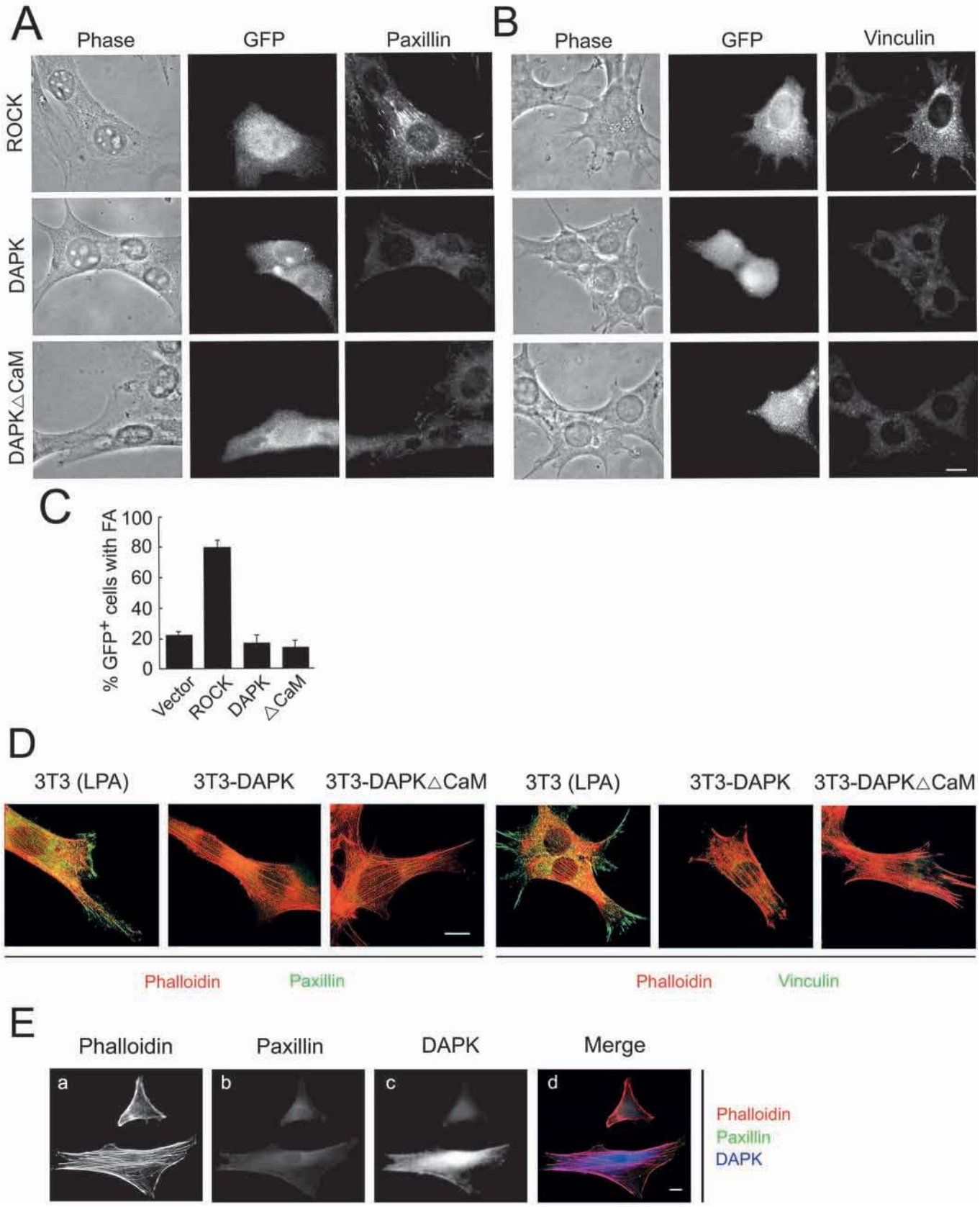
Thus, the spatial distribution of DAP kinase seems to play an important role in its cellular function.

DAP kinase mediates serum-induced stress-fiber formation

Having demonstrated that DAP kinase can play a positive role in the assembly of stress fibers, we investigated whether DAP kinase activity is required for the induction of stress fibers under certain physiological conditions. Using virus-infected NIH3T3 cells, as described above, we found that serum stimulation induced a rapid, robust formation of stress fibers in cells carrying the control vector (Fig. 5A). This induction of stress-fiber assembly, however, was greatly inhibited in cells carrying DAPK42A. This effect of DAPK42A was unlikely to result from nonspecific inhibition of ROCK, a kinase that is well known to participate in the induction of stress fibers by serum factors (Amano et al., 1997; Leung et al., 1996), because overexpression of DAPK42A did not alter the kinase activity of wild-type ROCK or its active mutant CAT (Amano et al., 1996) (Fig. 5B). DAPK42A was also incapable of blocking the activity of MLCK (Fig. 5C), further demonstrating the specificity of this mutant. To further elucidate the physiological role of DAP kinase in serum-induced stress-fiber formation, RNAi was used. NIH3T3 cells were transfected with a fluorophore-labeled, DAP-kinase-specific RNA oligonucleotide (DAPK siRNA) or a control siRNA. DAPK siRNA caused a 60% of reduction in the endogenous level of DAP-kinase protein without affecting that of a control protein (tubulin), whereas the control siRNA failed to knock down the expression of DAP kinase (Fig. 6A). Strikingly, cells receiving DAPK siRNA displayed fewer stress fibers in response to serum stimulation, in sharp contrast to the robust effects

Fig. 7. DAP kinase does not stimulate the formation of focal adhesions. (A,B) NIH3T3 cells co-transfected with various DAP kinase constructs and GFP at a ratio of 10:1 were serum starved for 6 hours and then stained with anti-paxillin (A) or anti-vinculin (B). The transfected cells are visualized by GFP fluorescence. (C) Quantitation of cells with focal adhesions in experiments as described in (A). The values shown are means ± s.d. from at least three independent experiments, and more than 300 individual cells were counted for each experiment. (D) NIH3T3 cells infected with recombinant retrovirus carrying DAP kinase or DAPKΔCaM were serum starved and then double stained with rhodamine-phalloidin (red) and anti-paxillin or anti-vinculin (green). Alternatively, NIH3T3 cells were treated with 200 ng ml^{-1} LPA for 15 minutes and then subjected to the same immunostaining. Images shown are the superimpositions of red and green fluorescence. (E) NIH3T3 cells transfected with DAP kinase were serum starved for 6 hours and then triple stained with rhodamine-phalloidin (a), anti-paxillin followed by FITC-conjugated anti-mouse secondary antibody (b) and anti-DAP-kinase followed by AMCA-conjugated anti-rabbit secondary antibody (c). The overlay of three images is shown in (d). Scale bars, 10 μm.

seen in cells without receiving siRNA or receiving a control siRNA (Fig. 6B). Thus, data from both RNA interference and dominant-negative interference strongly suggest an involvement of DAP kinase in serum-induced stress-fiber formation, prompting us to investigate whether DAP kinase's catalytic activity could be regulated in response to serum



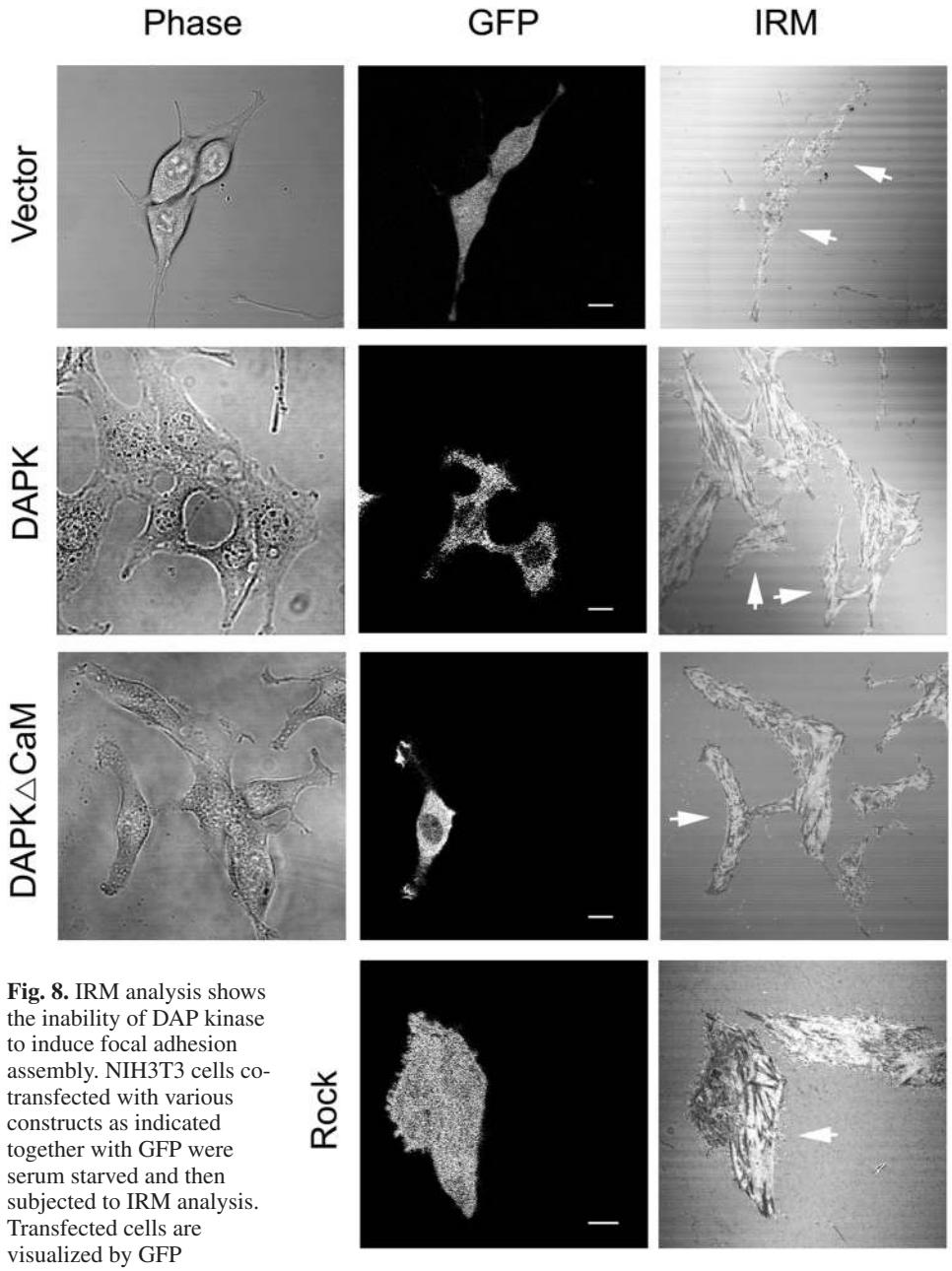


Fig. 8. IRM analysis shows the inability of DAP kinase to induce focal adhesion assembly. NIH3T3 cells co-transfected with various constructs as indicated together with GFP were serum starved and then subjected to IRM analysis. Transfected cells are visualized by GFP fluorescence and marked with white arrows in the IRM images. In cells transfected with control vector, DAP kinase or DAPKΔCaM, matched phase-contrast images are shown on the left to indicate the positions of the cells. Scale bars, 10 μm.

stimulation. Because antibody to DAP kinase competent for immunoprecipitations is unavailable, a Flag-tagged DAP kinase was transiently transfected into NIH3T3 cells to evaluate its activity in quiescent and serum-stimulated conditions. Although DAP kinase exhibited a considerable basal activity under serum-starved conditions, consistent with its capability of inducing stress fibers in quiescent cells, serum stimulation caused a rapid increase in its catalytic activity (Fig. 6C,D). Thus, in addition to its probable involvement in serum-induced stress-fiber formation, DAP kinase might also be capable of transducing cytoskeleton-reorganization signals elicited by serum factors.

DAP kinase does not stimulate focal-adhesion assembly

Induction of stress-fiber formation by various extracellular and intracellular factors is always accompanied by the stimulation of focal-adhesion assembly (Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996; Mackay et al., 1997; Arregui et al., 1998; Kiosses et al., 1999). We thus examined the ability of DAP kinase to promote the assembly of focal adhesions. NIH3T3 cells were transfected with vector for DAP kinase or DAPKΔCaM together with a GFP expression vector. The transfected cells were serum starved and then examined for the formation of focal adhesions by immunofluorescent staining with antibodies to several markers of focal adhesions, including paxillin (Fig. 7A), vinculin (Fig. 7B), FAK and β1-integrin (data not shown). Surprisingly, ectopic expression of either DAP kinase or DAPKΔCaM did not stimulate the assembly or stabilization of focal adhesions in serum-starved NIH3T3 cells. This inability of DAP kinase or DAPKΔCaM to promote focal-adhesion assembly is in sharp contrast with the function of ROCK (Fig. 7A-C) or its active mutant (data not shown), which led to strong immunofluorescent staining for paxillin (Fig. 7A), vinculin (Fig. 7B), FAK and β1-integrin (data not shown). To further demonstrate that the stabilization of stress fibers by DAP kinase does not cause a concomitant maintenance of focal adhesions, we used the virus-infected NIH3T3 cells described previously. Cells carrying DAP kinase or DAPKΔCaM virus were serum starved and then double stained with phalloidin and either anti-paxillin or anti-vinculin antibodies. This analysis revealed not only the stabilization of stress fibers without stimulating focal-adhesion formation by DAP kinase but also the appearance of very few focal adhesions in cells containing many stress fibers (Fig. 7D). As a control, NIH3T3 cells treated with lysophosphatidic acid (LPA) displayed strong staining patterns for both stress fibers and focal adhesions (Fig. 7D). Finally, to visualize directly and unequivocally the uncoupling of stress fibers and focal adhesions by DAP kinase, NIH3T3 cells transiently transfected with DAP kinase were serum starved and then triple stained with anti-DAP-kinase and anti-paxillin antibodies, and phalloidin (Fig. 7E). This analysis again revealed that cells

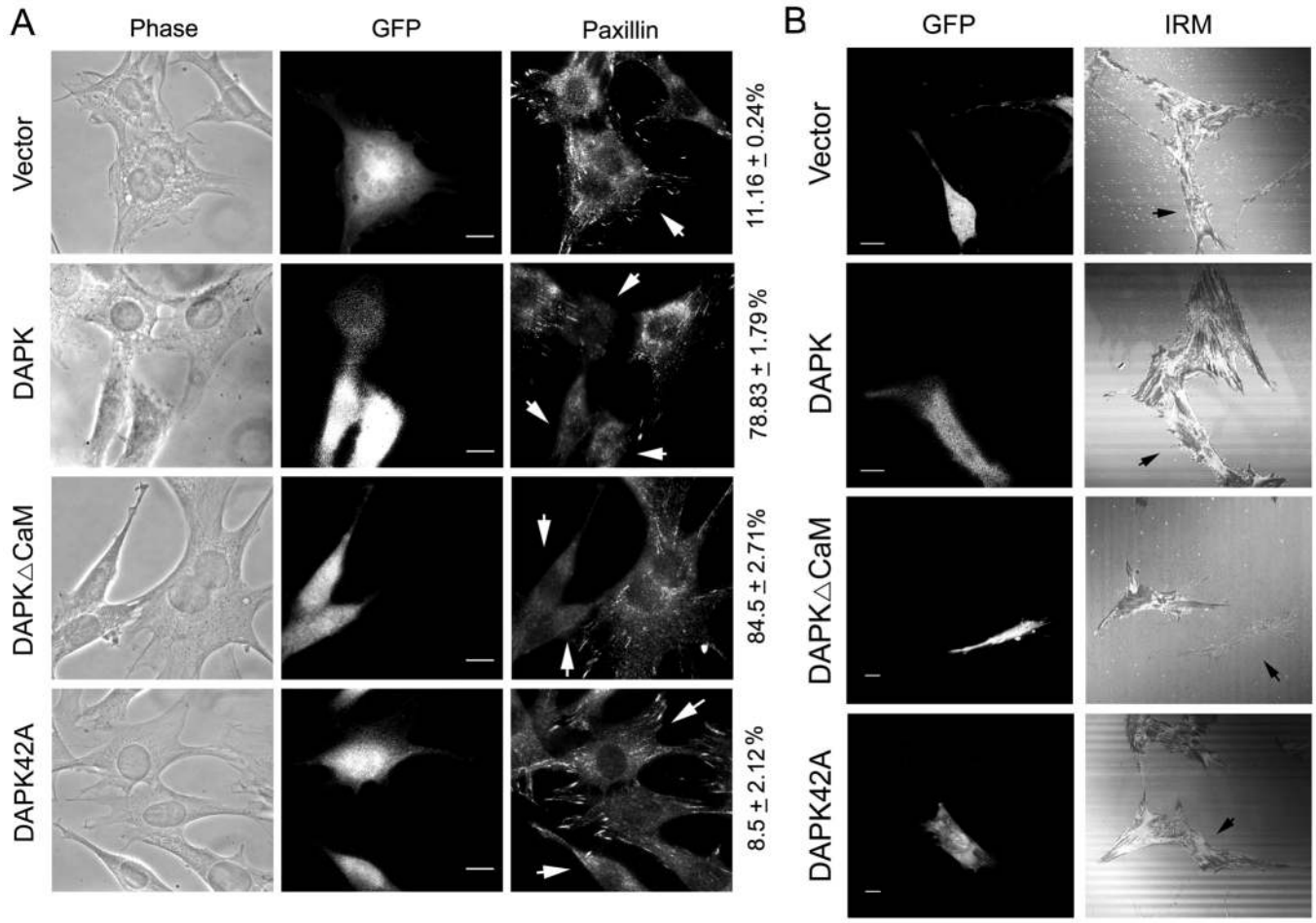


Fig. 9. DAP kinase promotes focal adhesion disassembly under serum-stimulated conditions. (A) NIH3T3 cells co-transfected with various DAP kinase constructs and GFP were cultured in serum-containing medium. Two days after transfection, cells were fixed and stained with anti-paxillin. The percentages of GFP-positive cells that display a weaker paxillin-staining pattern than the neighboring GFP-negative cells were quantified and are indicated on the right. The values shown are means ± s.d. from at least three independent experiments. (B) Cells as in (A) were directly analysed by IRM without fixation. (A,B) Transfected cells are visualized by GFP fluorescence and marked with arrows; (A) the phase-contrast image is also included. Scale bars, 10 μm.

overexpressing DAP kinase maintained their stress fibers but not their paxillin-containing focal adhesions. Thus, our findings clearly indicate the inability of DAP kinase to promote the assembly or maintenance of focal adhesions.

In addition to immunostaining analyses, we also used IRM analysis, which is an established method for examining the structural integrity of focal adhesions in living cells (Izzard and Lochner, 1976). Cells overexpressing ROCK, as indicated by their expression of GFP fluorescence, exhibited many large focal contacts, which are characterized by intense black streaks distributed in a radial direction throughout the cells and concentrated at cell edges (Fig. 8). In neighboring cells that did not express ROCK, however, the dark focal contacts were largely absent and significantly smaller. In cells expressing DAP kinase, DAPKΔCaM or control vector, the number and size of the dark contacts were virtually indistinguishable from those in neighboring cells that did not contain the transfected genes (Fig. 8), again demonstrating the inability of DAP kinase to stimulate focal adhesion assembly. Taken together, our results provide compelling evidence that the contractility generated by actin stress fibers is not always sufficient to drive the formation of focal adhesions and that assembly of stress fibers and focal adhesions could be regulated by separate mechanisms.

DAP kinase triggers disassembly of focal adhesions but not stress fibers in cells receiving serum growth factors

Having identified a unique feature of DAP kinase in the assembly or stabilization of stress fibers without a concomitant induction of focal adhesions in quiescent cells, we next determined its effect on cells grown in serum-containing medium. NIH3T3 cells transiently co-transfected with DAP kinase and GFP were cultured in the presence of serum and then stained with anti-paxillin (Fig. 9A) or anti-vinculin (data not shown). Surprisingly, cells overexpressing DAP kinase exhibited both reduced numbers and weak staining intensity of focal adhesions (Fig. 9A), in sharp contrast to the neighboring cells without expressing the exogenous DAP kinase. This disassembly of focal adhesions was also observed in cells expressing DAPKΔCaM, but not in those expressing DAPK42A (Fig. 9A). Furthermore, IRM analysis demonstrated a nearly complete loss of the large, dark focal

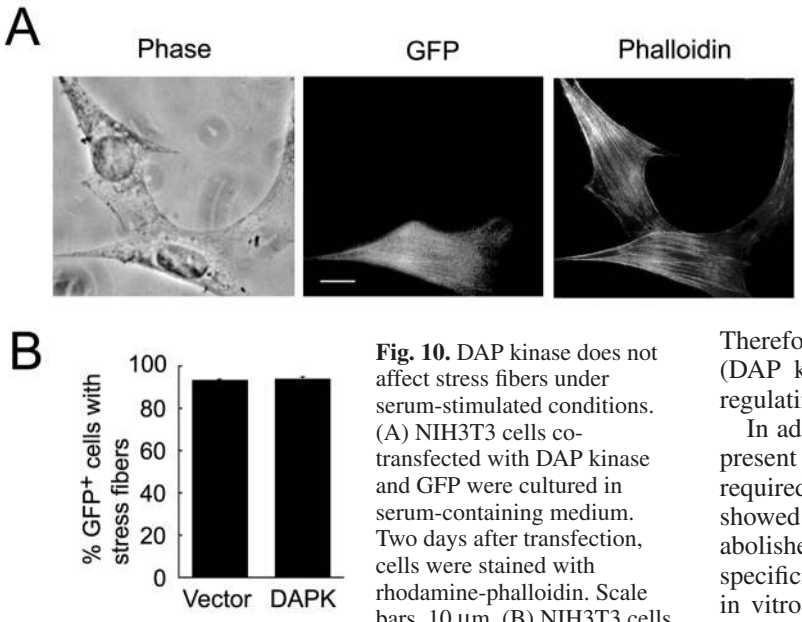


Fig. 10. DAP kinase does not affect stress fibers under serum-stimulated conditions. (A) NIH3T3 cells co-transfected with DAP kinase and GFP were cultured in serum-containing medium. Two days after transfection, cells were stained with rhodamine-phalloidin. Scale bars, 10 μm. (B) NIH3T3 cells transfected with DAP kinase or control vector together with GFP were cultured and stained as in (A). The percentages of GFP-positive cells containing stress fibers were quantified and plotted.

adhesion images at periphery of the cell that overexpressed DAP kinase, whereas neighboring cells that did not express DAP kinase showed many black streaks, characteristic of focal adhesions (Fig. 9B). Similar results were observed with cells expressing DAPKΔCaM. However, in cells receiving control vector or DAPK42A, their IRM images were not significantly differed from those of neighboring, untransfected cells (Fig. 9B). Despite DAP kinase being efficient at promoting focal adhesion disassembly, it did not cause the disassembly of actin stress fibers under the same conditions (Fig. 10A,B). Altogether, these results indicate that DAP kinase causes an uncoordinated regulation of focal adhesions and stress fibers in both serum-starved and stimulated cells, and imply that DAP kinase might perturb the balance between contractile and adhesion forces.

Discussion

In this report, we have identified a novel function of DAP kinase as a potent inducer of cytoskeletal reorganization. We show that DAP kinase can stabilize stress fibers in quiescent cells. This effect requires its catalytic activity but not an intact death domain, and is probably the result of the increased MLC phosphorylation. However, unlike the distantly related MLCK (Totsukawa et al., 2000; Katoh et al., 2001), DAP kinase is dispensable for the assembly of cortical actin filaments. Recent studies indicated that ROCK is responsible for the assembly of stress fibers in the center of cells, whereas MLCK functions in the formation of cortical actin bundles (Totsukawa et al., 2000; Katoh et al., 2001). Such different regulation of actin filaments is partly attributed to the intracellular localizations of ROCK and MLCK, being in the central and peripheral regions of cells, respectively (Totsukawa et al., 2000). Endogenous DAP kinase is enriched in the central region of cells and is essentially undetectable at the cell periphery, which might explain its spatial specificity in the assembly of actin filaments. Although DAP kinase and ROCK are both involved in the assembly of central stress fibers, the morphologies of stress fibers induced by the two kinases are somewhat different. The stress fibers developed in DAP-kinase- or DAPKΔCaM-overexpressing cells are thinner and parallel, whereas stellar stress fibers are formed in cells expressing an active form of ROCK (Leung et al., 1996; Amano et al., 1997; Ishizaki et al., 1997). Therefore, the three different kinases that phosphorylate MLC (DAP kinase, MLCK and ROCK) display distinct roles in regulating the assembly of actin filaments.

In addition to stabilizing stress fibers in quiescent cells, we present compelling evidence indicating that DAP kinase is also required for serum-induced formation of stress fibers. We first showed that dominant-negative interference of this kinase abolishes stress-fiber formation induced by serum. The specificity of the dominant-negative mutant was verified using in vitro kinase assays. Because these assays might not fully reflect situations occurring in vivo, siRNA-mediated knockdown of DAP kinase was also used to provide an independent line of evidence. Consistent with a role in serum-induced stress-fiber formation, the catalytic activity of DAP kinase is increased in response to serum stimulation. However, our demonstration that DAP kinase functions as a positive mediator of serum-induced stress fibers does not preclude a similar role for other kinases, such as ROCK, in this process. Perhaps stress fibers only form when the intracellular level of phosphorylated MLC reaches a certain threshold and inactivation of either ROCK or DAP kinase might reduce the level of phosphorylated MLC to a level that is below this threshold, thus blocking stress fiber formation. Thus, DAP kinase and ROCK might act cooperatively to regulate stress fiber formation in response to serum stimulation.

Induction of MLC phosphorylation and stress-fiber formation by various factors and proteins is always accompanied by the assembly of focal adhesions (Ridley and Hall, 1992; Leung et al., 1996; Amano et al., 1997; Totsukawa et al., 2000; Katoh et al., 2001), and MLC phosphorylation was reported to be both sufficient and necessary for the formation of stress fibers and focal adhesions (Totsukawa et al., 2000). However, as an exception, we found that the induction of MLC phosphorylation and stabilization of stress fibers by DAP kinase does not lead to a concomitant stimulation of focal adhesion assembly. Furthermore, in cells cultured in serum-containing medium, DAP kinase is even capable of inducing focal adhesion disassembly without affecting stress fibers. These results indicate that DAP kinase triggers an uncoupling of the formation of stress fibers and focal adhesions in both serum-starved and stimulated cells, and imply that the contractility generated by stress fibers is not the only determining factor for the assembly of focal adhesion complexes. Indeed, a similar disassembly of focal adhesions but not stress fibers was reported by the activation of the PIX-GIT1 complex. In this case, GIT1 interacts directly with paxillin, which leads to a disengagement of paxillin from, and consequent disassembly of, focal complexes (Zhao et al., 2000). Uncoupling of stress-fiber and focal-adhesion formation is also evident in BDM-treated $FAK^{-/-}$ cells, which display

normal focal adhesions despite lacking stress fibers (Chen et al., 2002). How DAP kinase leads to the uncoordinated regulation of stress fibers and focal adhesions, however, is currently unclear. Perhaps MLC is not the only substrate of DAP kinase and phosphorylation of other unidentified substrates might play an inhibitory role in the assembly of focal adhesions without affecting stress fibers. Assembly of focal adhesion complexes requires the clustering of integrins, which can be induced from both inside cells (by bundling of associated actin filaments) and outside cells [by binding of integrins to extracellular matrix (ECM)]. Notably, we recently found that DAP kinase inhibits integrin activity and cell attachment to ECM (Wang et al., 2002).

It is noteworthy that the cytoskeletal reorganization effect of DAP kinase is not a consequence of apoptosis and occurs prior its onset. When cells overexpressing DAP kinase or DAPKΔCaM were assayed for the formation of stress fibers and focal adhesions, they displayed normal nuclear and cell morphology and do not exhibit increased DNA fragmentation. However, prolonged culture of cells overexpressing DAP kinase or DAPKΔCaM resulted in apoptosis. These observations lead to a hypothesis that DAP-kinase-induced cytoskeletal reorganization predisposes cells to apoptosis. Owing to its ability to trigger an uncoordinated regulation of stress fibers and focal adhesions, DAP kinase might cause an increase in the contractile force without a compensatory enhancement in the adhesive strength generated by focal adhesions. Such a perturbation of the balance between contractility and adhesion might lead to the detachment of cells from ECM and/or downregulation of ECM survival signals, thereby triggering apoptosis. Consistent with this notion, we recently found that DAP kinase induces an anoikis-type apoptosis by inside-out inactivation of integrin (Wang et al., 2002). Thus, the uncoordinated regulation of stress fibers and focal adhesions by DAP kinase might play a causative role in its apoptotic effects.

In addition to identifying a role of DAP kinase in cytoskeletal remodeling, we present several lines of evidence indicating that DAP kinase is a bona fide MLC kinase in vivo. First, the K_m value of DAP kinase for MLC indicates MLC is a reasonable substrate for DAP kinase (data not shown). Second, DAP kinase can directly interact with MLC. Third, overexpression of DAP kinase increases the level of phosphorylated MLC, whereas dominant-negative inhibition of this kinase blocks MLC phosphorylation in vivo. Because DAP kinase is the prototype of the DAP kinase family (Cohen and Kimchi, 2001), MLC is probably a common in vivo substrate for many, if not all, DAP-kinase-related proteins. Accordingly, all DAP kinase family members are capable of phosphorylating MLC in vitro (Inbal et al., 2000). Furthermore, ZIP kinase induces MLC phosphorylation and actin filament reorganization in vivo (Murata-Hori et al., 2001). Because each of these kinases was reported to promote apoptosis in a kinase-activity-dependent manner (Kawai et al., 1998; Sanjo et al., 1998; Inbal et al., 2000), cytoskeletal rearrangement caused by MLC phosphorylation might account for at least one common mechanism of apoptosis induced by DAP kinase family proteins. Consistent with this notion, most of the family members were reported to be capable of inducing apoptotic morphological changes, such as membrane blebbing and cell rounding (Kawai et al., 1998; Sanjo et al., 1998; Inbal et al., 2002), which are probably the consequences of MLC phosphorylation and perturbation of the balance between contractility and adhesion, respectively.

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