Myotonic Dystrophy Kinase-Related Cdc42-Binding Kinase Acts as a Cdc42 Effector in Promoting Cytoskeletal Reorganization

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The Rho GTPases play distinctive roles in cytoskeletal reorganization associated with growth and differentiation. The Cdc42/Rac-binding p21-activated kinase (PAK) and Rho-binding kinase (ROK) act as morphological effectors for these GTPases. We have isolated two related novel brain kinases whose p21-binding domains resemble that of PAK whereas the kinase domains resemble that of myotonic dystrophy kinase-related ROK. These ~190-kDa myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKs) preferentially phosphorylate nonmuscle myosin light chain at serine 19, which is known to be crucial for activating actin-myosin contractility. The p21-binding domain binds GTP-Cdc42 but not GDP-Cdc42. The multidomain structure includes a cysteine-rich motif resembling those of protein kinase C and n-chimaerin and a putative pleckstrin homology domain. MRCKα and Cdc42V12 colocalize, particularly at the cell periphery in transfected HeLa cells. Microinjection of plasmid encoding MRCKα resulted in actin and myosin reorganization. Expression of kinase-dead MRCKα blocked Cdc42V12-dependent formation of focal complexes and peripheral microspikes. This was not due to possible sequestration of the p21, as a kinase-dead MRCKα mutant defective in Cdc42 binding was an equally effective blocker. Coinjection of MRCKα plasmid with Cdc42 plasmid, at concentrations where Cdc42 plasmid by itself elicited no effect, led to the formation of various constitutively active forms of Cdc42 in the heterotrimeric G-protein-coupled yeast pheromone Kss1/Fus3 mitogen-activated protein kinase pathway (47, 54). Similarly, mammalian Cdc42 and Rac1 have also been found to have nuclear signaling roles through the JNK/SAPK mitogen-activated protein kinase pathway (13, 39). Several reports have also implicated PAKs in these events (5, 8, 53), suggesting a parallel conservation of components in these signaling events among eukaryotes. In mammalian cells, expression of various constitutively active forms of α-PAK results in disassembly of focal complexes and stress fiber dissolution, suggesting that these kinases also have morphological roles (34). ROKs also have effects on morphology, with their overexpression enhancing the formation of stress fibers and focal adhesion complexes (1, 20, 30). This effect of ROKs may be mediated by their inhibition of myosin phosphatase through specific phosphorylation of its myosin-binding subunit, which increases the phosphorylation state of myosin light chain (23). Alternatively, ROKs may also activate myosin through direct phosphorylation of myosin light chain (2). These results suggest that a diverse network of p21 targets, in particular kinases, is involved in both nuclear and cytoskeletal control (32). The use of mutants of Rac1 and Cdc42Hs has also revealed different pathways utilizing distinctive effectors for morphological as well as transcriptional activation (21, 27, 51). Apart from PAKs, the p21 binding assay has revealed the presence of multiple proteins of 180 to 200 kDa in a variety of rat tissues which bind Cdc42Hs/Rac1 (36). We have purified several ~180-kDa Cdc42/Rac1-binding proteins from rat brain and liver which turned out to be identical to IQGAP isoforms

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The Ras-related p21 Rho subfamily GTPases are implicated in actin reorganization, although the exact mechanisms involved remain largely obscure (48). In Swiss 3T3 fibroblasts, introduction of Cdc42 into cells resulted in filopodial formation (24, 41), while Rac1 and RhoA give rise to lamellipodia and stress fibers, respectively (43, 44). Apart from cell morphology, the Rho p21s are also involved in processes such as cell growth, cytokinesis, activation of transcription factors, and cell cycle progression (13, 14, 39, 40, 42). An important step toward understanding the biochemical mechanisms by which these p21s exert their diverse cellular effects is to identify and characterize interacting proteins which mediate the actions of a particular p21. To date, a large number of proteins which interact with Rho p21s have been reported. These include regulatory proteins such as GTPase-activating proteins, guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors and an increasing number of kinases and nonkinases (32, 48). Most of these molecules have multidomain structures (10, 26, 32), suggesting the existence of a wide range of multimeric complexes in regulating signaling pathways underlying cell morphology and other related cellular activities. Such complexity has been shown in lower organisms such as Saccharomyces cerevisiae in which normal polarized cell growth and cell shape changes are accomplished by the interaction of Cdc42p with several proteins, including Cdc24 (a guanine nucleotide exchange factor), Ste20p kinase, and actin-binding protein (28, 52). In mammalian cells, input from other signaling pathways can also be implicated in the p21 functions; e.g., phosphatidylinositol 3-kinase can mediate signaling from Ras to Rac1 (45). In searching for potential targets of the p21 Rho family, we and others have identified p21-activated kinases (PAKs) which specifically interact with GTP-Cdc42/Rac1 (36) and the RhoA-binding kinases (ROKs) (19, 30, 31, 38). Interaction of PAK with p21 in vitro results in kinase activation (36, 37). This novel activation process has led us and others to postulate that the yeast homolog of mammalian PAK, Ste20p, may act downstream of Cdc42p in the heterotrimeric G-protein-coupled yeast pheromone Kss1/Fus3 mitogen-activated protein kinase pathway (47, 54). Similarly, mammalian Cdc42 and Rac1 have also been found to have nuclear signaling roles through the JNK/SAPK mitogen-activated protein kinase pathway (13, 39). Several reports have also implicated PAKs in these events (5, 8, 53), suggesting a parallel conservation of components in these signaling events among eukaryotes. In mammalian cells, expression of various constitutively active forms of α-PAK results in disassembly of focal complexes and stress fiber dissolution, suggesting that these kinases also have morphological roles (34). ROKs also have effects on morphology, with their overexpression enhancing the formation of stress fibers and focal adhesion complexes (1, 20, 30). This effect of ROKs may be mediated by their inhibition of myosin phosphatase through specific phosphorylation of its myosin-binding subunit, which increases the phosphorylation state of myosin light chain (23). Alternatively, ROKs may also activate myosin through direct phosphorylation of myosin light chain (2). These results suggest that a diverse network of p21 targets, in particular kinases, is involved in both nuclear and cytoskeletal control (32). The use of mutants of Rac1 and Cdc42Hs has also revealed different pathways utilizing distinctive effectors for morphological as well as transcriptional activation (21, 27, 51). Apart from PAKs, the p21 binding assay has revealed the presence of multiple proteins of 180 to 200 kDa in a variety of rat tissues which bind Cdc42Hs/Rac1 (36). We have purified several ~180-kDa Cdc42/Rac1-binding proteins from rat brain and liver which turned out to be identical to IQGAP isoforms
isolated by others (18, 25). We now report the isolation and characterization of MRCKs, a novel family of ~190-kDa serine/threonine kinases highly related to the myotonic dystrophy kinase (7, 15) and ROKs (19, 30, 31, 38), which interact strongly with the GTP-bound form of Cdc42. These kinases also contain a cysteine-rich domain capable of binding to phorbol ester and a putative pleckstrin homology (PH) domain. The possible involvement of these kinases as Cdc42 effectors in cytoskeletal reorganization is also presented.

MATERIALS AND METHODS

Screening and expression of MRCKα and β. A agt11 human brain cDNA library (Clontech) was used for expression screening with [γ32P]GTP-glutathione-3-transferase (GST)-Cdc42Hs as previously described (15). The 373-bp positive cDNA clone encoding the 124-amino-acid residues and its deleted and mutated derivatives were subcloned into pGEX vectors for expression and p21 binding analysis. For isolating the full-length clones, a rat brain cDNA library (Stratagene) was used. The full-length MRCKα was derived from an 8-kb cDNA clone containing the entire coding sequence. The MRCKβ sequence was derived from three overlapping clones. Full-length MRCKα (see below) was subcloned into pBPK-GST vector for expression in the baculovirus system (Clontech). The GST fusion protein was purified through a glutathione-Sepharose column and used for kinase assays with various substrates. For expression in mammalian cells, MRCKs were subcloned into pBlueScript SK vector into either plasmid pXJ40-HA or plasmid pXJ40-FLAG (34). A BamHI/Psil-cut digested PCR product of the 5' end corresponding to the N-terminal kinase domain was obtained by using a 5' primer (5'GGCGATACCAATGTGTCGGAGAAGTGCGG3') and a 3' primer (5'CCTGCGAAGCCTGCTG3') and ligated to the BamHI/Psil-cut pXJ40 vector to generate the kinase domain construct MRCKα-1-473. Full-length MRCKα-1-1732 was obtained by replacing all XhoI/KpnI fragment of this subclone by a longer 6-kb XhoI/KpnI fragment from the full-length SK vector. For MRCKΔP, an in-frame deletion of an EcoRV/Nhel (blunted) (residues 1117 to 1181) was used. For mutagenesis, a two-step PCR protocol (34) with VENT polymerase (New England Biolabs) was used. The p21-binding-defective mutant (MRCKαK106A) was obtained with primers GTTAAAATGTTGGGC-3'/T3 and 5’GCCATACGACGATGCTCCTGACCTG-3'/T7, and the kinase-dead mutant (MRCKαK625A) was obtained with primers 5’GGCCATACGAAATACCTATC123/C7 and 5’GCCATGGCAAATACTTTATC3’. The 373-bp EcoRI fragment of MRCKα-1-473 was obtained by replacing an XhoI/KpnI fragment from the full-length SK vector. For MRCKΔP, an in-frame deletion of an EcoRV/Nhel (blunted) fragment (residues 1117 to 1181) was used. For mutagenesis, a two-step PCR protocol (34) with VENT polymerase (New England Biolabs) was used. The p21-binding-defective mutant (MRCKαK106A) was obtained with primers GTTAAAATGTTGGGC-3'/T3 and 5’GCCATACGACGATGCTCCTGACCTG-3'/T7, and the kinase-dead mutant (MRCKαK625A) was obtained with primers 5’GGCCATACGAAATACCTATC123/C7 and 5’GCCATGGCAAATACTTTATC3’.

RESULTS

Identification of a family of Cdc42Hs-binding proteins. (A) Deduced amino acid sequence of a human brain partial cDNA clone isolated by expression screening with [γ32P]GTP-Cdc42. GST fusion proteins were made with wild-type (construct 1), deleted (constructs 2 and 3), or mutated (construct 4) variants. In construct 4, two histidines (underlined) were mutated to alanine. Binding of [γ32P]GTP-Cdc42 was performed as described previously (35). (B) Nucleotide-dependent binding of two related rat Cdc42-binding proteins. Nicotinucleotide filters with 50 ng of GST fusion protein containing the binding domain of human MRCKα (residues 1 to 124; lane 1), rat MRCKβ (residues 1569 to 1600; lane 2), and pPAK (residues 67 to 150; lane 3) were assayed for binding with a 32P-phosphorylated Cdc42Hs (from pGEX-2TK) exchanged with either GTP-S or GDP (31). (C) Consensus sequence of Cdc42-binding motifs of different proteins (9), that DOSPR (5 μM) was used. For immunoprecipitation experiments, cells grown in a 100-mm-diameter dish were transfected and incubated for 16 h before harvest. For immunofluorescence studies, HeLa cells were plated onto glass chamber slides (Nunc), transfected in the presence of 5% serum, and fixed after 16 h of incubation. For microinjection, HeLa cells maintained in minimal essential medium in the presence or absence of 10% fetal bovine serum were used. Subconfluent cells plated on coverslips for 48 h were microinjected with different constructs (50 ng/ml except where indicated otherwise), by using an Eppendorf micromanipulator system. Two to four hours after injection, cells were fixed with 4% paraformaldehyde and incubated in phosphate-buffered saline-0.5% Triton X-100 for 2 h at 25°C with the combination of various primary antibodies at the following dilutions: anti-HA (12CA5) or anti-FLAG (IBI) MAb, 5 μg/ml; antivinculin MAb (hVIN-1; Sigma), 1:300; antipaxillin MAb (Transduction Laboratories), 2 μg/ml; anti-myosin light chain MAb (Sigma), 1:100. Fluorescein isothiocyanate-conjugated phorphoamino acid analysis (36) or complete Lys-C digestion. A single phosphorylation peak obtained from high-pressure liquid chromatography was analyzed by peptide sequencing with simultaneously radioactive detection of each residue. For detecting kinase activity of MRCKs in overexpressed COS-7 cells, transfected cells were extracted with lysis buffer containing 25 mM HEPES (pH 7.3), 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM sodium β-glycerophosphate, 1 mM sodium vanadate, 0.5% Triton X-100, 5% glycerol, and freshly added 5 mM sodium dodecyl sulfate sample buffer. After a brief spin in an Eppendorf tube, the reaction mixture was run on a 10% polyacrylamide gel and transferred onto a nitrocellulose filter for radioactive imaging and subsequent detection of expressed proteins by a rabbit anti-HA antibody (BabCo). 32P phosphorilation of MLC-2 was quantified with a Molecular Dynamics Phosphor-Imager.

Transfection and microinjection. COS-7 cells and HeLa cells maintained in 10% fetal bovine serum were transfected essentially as described previously (30) except...
Cdc42, we obtained a partial cDNA from a human brain cDNA library. This cDNA fragment, a shorter AseI/EcoRI-deleted fragment, and a PCR fragment flanking the putative p21-binding site when expressed as GST fusion proteins all bound GTP

\[ \text{GTP}_{\text{G}} \text{Cdc42} \] but not GDP-Cdc42 (Fig. 1A and B). Double mutation of the conserved histidines led to abolition of binding. Weak binding was also observed to GTP-Rac1 but not to GTP-RhoA (data not shown). The binding domain is conserved.

**FIG. 2.** Sequence of a family of Ser/Thr kinases containing a Cdc42-binding domain and other functional domains. (A) Deduced amino acid sequences of rat MRCKα and MRCKβ. Regions in boldface represent, in order, kinase, cysteine-rich (CR), PH, and p21 GTPase-binding (GBD) domains. In MRCKα, the region underlined is identical, apart from an initial L3V, to the human sequence shown in Fig. 1A. Domain organization of MRCKs, myotonic dystrophy kinase (DMK), and ROKα, along with percent identities of related domains, is also shown. (B) Kinase domains of MRCKα, MRCKβ, myotonic dystrophy kinase (DMK), and ROKα. (C) PH domains in MRCKα, MRCKβ, ROKα, and pleckstrin N terminus (Pleck N). Amino acids identical to the most commonly occurring consensus sequence in PH domains (in boldface and uppercase) are marked with asterisks. (D) Cysteine-rich domains of MRCKs, PKCa, and n-chimaerin (n-CHIM). Conserved residues (17) are indicated by asterisks. The保守的残基（17）由星标表示。
served in the two rat isoforms isolated from further screening and resembles other p21-binding motifs of this class (9, 36) (Fig. 1C) but not the RhoA-binding sequence of ROK or protein kinase N (PKN) (3, 30, 50).

Identification of MRCKs. Two related full-length cDNAs were isolated upon subsequent screening of a rat brain cDNA library. The N termini of the predicted proteins (Fig. 2A) begin with a kinase domain (Fig. 2B) exhibiting 68% identity to the human myotonic dystrophy kinase. These kinases were designated MRCKα and β. The kinase domains were followed by an extended α-helix, with coiled-coil features (residues 450 to 950 in MRCKα) and a highly conserved region (residues 810 to 860 in MRCKα) which has some homology to nonmuscle myo-

FIG. 2—Continued.
osin heavy chain and rat nestin (29). Both isoforms of ~190 kDa contain the p21-binding motif near the C-terminus, with the domain organization of these kinases being quite different from that of ROKα (Fig. 2A). A cysteine-rich domain (Fig. 2D) and a pleckstrin-like domain (Fig. 2C) occur between the kinase domain and the p21-binding motif.

**Biochemical characterization of MRCKs.** The expression of these p190 kinases was examined in protein extracts by Cdc42-GTP binding and Western blot analysis using polyclonal antibodies against the p21-binding domain of human MRCKα. As reported previously, major Cdc42 binding occurs in regions from around 180 to 200 and 62 to 68 kDa; the latter corresponds to PAK isoforms (36). The larger Cdc42-binding proteins probably include MRCKα and -β. Western analysis revealed 180- to 200-kDa proteins, present at higher levels in the brain and kidney (Fig. 3A). High levels of immunoreactivity were detected in lung, in the pellet fraction (data not shown). The Cdc42-binding pattern did not correlate well with p180-200 immunoreactivity in different tissues, possibly because of the presence of other MRCK isoforms or Cdc42-binding proteins such as IQGAPs, which have similar molecular sizes (18, 25). On Northern blot analysis, the 10-kb MRCKα mRNA was highly enriched in the brain and lung and present in lower levels in other tissues (Fig. 3B). MRCKβ mRNA was expressed in all tissues examined and at highest levels in lung and kidney. Both mRNAs were also expressed in epithelial HeLa cells (data not shown).

MRCKα when expressed as a GST fusion protein was found to phosphorylate serine/threonine residues of several

![FIG. 4. Cellular localization of MRCKα and the effects of Cdc42V12. HeLa cells grown in minimal essential medium with 10% fetal bovine serum were transfected with pXJ40-FLAG plasmids encoding either MRCKα alone or MRCKαΔPH (the latter with the PH domain deleted). Cells were fixed with 4% paraformaldehyde and stained with anti-FLAG antibody after 16 h. For cotransfection experiments, plasmid encoding FLAG-tagged MRCKα or MRCKαΔPH was cotransfected with plasmid encoding HA-tagged Cdc42V12. Cells were fixed and doubly stained with antibodies against FLAG for MRCKα and HA for Cdc42V12.](http://mcb.asm.org/)

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substrates, including myelin basic protein, histone H1, and its own binding domain (hBF-1) in vitro, but was especially active toward nonmuscle myosin regulatory light chain (MLC-2), the latter being phosphorylated at serine 19 (Fig. 3C). Immunoprecipitated HA-MRCKα did not exhibit elevated activity when cotransfected with Cdc42V12 (Fig. 3D). Similar results were obtained with the recombinant full-length GST-MRCKα (data not shown), indicating that in-

FIG. 5. MRCKα affects the organization of cellular structures. HeLa cells grown on coverslips were microinjected with a plasmid encoding HA-tagged wild-type MRCKα (a and b), kinase-dead MRCKαK106A (c and d), or kinase domain alone (e to h). Two hours after incubation, cells were fixed and stained with anti-HA antibody (a and c) or doubly stained with phalloidin (b, d, and e) or antibodies against myosin light chain (f), vinculin (g), or tubulin (h). Arrows indicate the injected cells located by HA staining (not shown in panels e to h). Bar = 10 μm.
FIG. 6. MRCKα potentiates the effects of Cdc42 on microspike formation. (A) Kinase-dead MRCKα blocks Cdc42-mediated effects on focal complexes and morphology. Serum-starved HeLa cells were injected with plasmid encoding FLAG-tagged kinase-dead/p21-binding-deficient mutant MRCKα<sup>K106A,H1579A,H1581A</sup> (50 ng/μl); 3 h later, these preinjected cells (a, b, e, and f) and uninjected control cells (c, d, g, and h) were injected with plasmid pXJ40-HA (50 ng/μl) encoding Cdc42<sup>V12</sup> (a to d) or Rac1<sup>V12</sup> (e to h). Cells were fixed and stained with antibodies against FLAG (a and e), HA (c and g), or paxillin (b, d, f, and h) after incubating for 2 h. Essentially similar results were obtained with kinase-dead MRCKα<sup>K106A</sup>. (B) Morphological effect of expression of MRCKα and limiting amounts of Cdc42. HeLa cells grown in serum-containing medium were injected with plasmids encoding FLAG-tagged Cdc42 (5 ng/μl) together with plasmid encoding either HA-tagged wild-type MRCKα (a), MRCKα<sup>K106A</sup> (b), p21-binding-defective MRCKα<sup>H1579A,H1582A</sup> (c), or ROKα (d) at 50 ng/μl. Cells incubated for 2 h were fixed and stained with anti-HA antibody. Bar = 10 μm. (C) Time-lapse phase-contrast microscopy of HeLa cells coinjected with plasmids encoding MRCKα and Cdc42 as in panel B. Morphological changes in a typical coinjected cell are shown up to 4 h after the coinjection. Cells 4 h after injection with plasmids encoding MRCKα (50 ng/μl) alone, Cdc42 (5 ng/μl) alone, or Cdc42<sup>V12</sup> (50 ng/μl) are included for comparison.
Interaction with Cdc42 was not essential for kinase activation. The mutant MRCKαK106A, with a substitution of the critical lysine in the kinase domain, exhibited no detectable kinase activity.

The cysteine-rich domain in both isoforms resembles those of PKC and chimaerins and was capable of binding to [3H] phorbol myristic acetate in a lipid-dependent manner (data not shown).
Cellular localization of MRCKα and the effect of Cdc42V12. In transfected HeLa cells, expressed FLAG-MRCKα showed a dispersed punctate cytoplasmic distribution and a more intense staining along the cell periphery, especially at the leading edge and cell-cell junction. Cotransfection with Cdc42V12 led to a typical Cdc42-type morphology, and MRCKα was found to colocalize with Cdc42V12, particularly at the cell-cell junction and periphery, which contained numerous protrusions (Fig. 4, left panels). As PH domains can interact with lipids and the cytoskeleton, we also studied the effects of MRCKαΔPH, a construct with the PH domain deleted. Cells transfected with MRCKαΔPH plasmid showed a more even cytoplasmic distribution of the kinase. When cotransfected with Cdc42V12, both MRCKαΔPH and p21 remained largely dispersed within the cytoplasm, and the typical Cdc42-type morphology was not produced (Fig. 4, right panels). These results suggest that the PH domain is important for the correct localization of MRCKα and that MRCKα may be associated with producing a Cdc42 phenotype (possibly acting as a dominant-negative mutant).

Microinjection of MRCKα affects cellular structures. To investigate whether MRCKα had a direct effect on morphology, we microinjected HeLa cells with plasmids encoding MRCKα and various derivatives. Expression of wild-type MRCKα enhanced the formation of stress fibers, some of which exhibited a crisscross pattern (Fig. 5a and b). The kinase domain alone (which is constitutively active) elicited gross changes in actin- and myosin-containing structures involving marked actin condensation (Fig. 5e and f). Some increase in focal complexes was observed in focal complexes were seen (Fig. 5g), but microtubules were unaffected (Fig. 5h). The action of MRCKα in promoting formation of stress fibers was reminiscent of the action of the related ROKα. However, MRCKα notably differed from ROKα in that its kinase-dead mutant (MRCKαK106A) did not promote dissolution of existing stress fibers (Fig. 5c and d). The MRCKα promotion of stress fibers was also not affected by the dominant-negative ROKαK112A (not shown, being very similar to Fig. 5a and b), indicating that MRCKα did not act via ROKα. These results show that although overexpressed MRCKα can mimic some effects of ROK through the presence of a kinase domain which is highly homologous among a family of diverse proteins, MRCKα appears to have a role different from that of the Rho-binding ROK.

MRCKα modulates Cdc42-dependent morphology. We then examined whether MRCKα could have a role in the morphological effects promoted by Cdc42. In HeLa cells, these morphological effects include microspike formation and production of peripheral focal complexes readily observed 2 h after injection of Cdc42V12 plasmid (50 ng/μl) (Fig. 6A, panels c and d). When these cells were first injected with plasmid encoding kinase-dead MRCKαK106A (using this as a putative dominant-negative mutant) 3 h before the injection of Cdc42V12, these morphological effects were not seen. This blocking effect of the kinase-dead MRCKαK106A mutant was not due to its possible sequestration of Cdc42V12, since prior expression of the kinase-dead and Cdc42-binding-deficient MRCKαK106A,H1579A/H1582A mutant was as effective in inhibiting the morphological action of Cdc42V12 (Fig. 6A, panels a and b). This MRCKα mutant had no effect on Rac1-induced focal complexes or cell spreading (Fig. 6A, panels e to h), showing that it specifically affected Cdc42 actions.

We next investigated the functional relationship of MRCKα to its p21 partner Cdc42, adopting an approach similar to one recently used to study the effects of POR1, a Rac1-binding protein, on cytoskeletal reorganization (49). We first established that injection of low concentrations (5 ng/μl) of wild-type Cdc42 plasmid was phenotypically ineffective in inducing morphological changes in HeLa cells and subsequently coinjected MRCKα and Cdc42 plasmids. This led to an enhanced formation of cellular extensions and microspikes with a marked redistribution of MRCKα to cortical regions, especially at the tip of the former structures (Fig. 6B, panel a). Coinjection of Cdc42 plasmid with plasmids encoding kinase-dead MRCKαK106A, Cdc42-binding-deficient MRCKαH1579A/H1582A and ROKα (Fig. 6B, panels b to d) led to no such enhanced formation of peripheral structures. These results indicate that both kinase and Cdc42-binding domains of MRCKα are required for its effects on Cdc42 functions.

Injected cells were then subjected to time-lapse analysis. When injected with a low concentration (5 ng/μl) of Cdc42 plasmid, cells showed very little change even after 4 h. Higher concentrations (50 ng/μl) of activated Cdc42V12 plasmid led to the appearance of short microspikes (Fig. 6C, bottom row). When MRCKα plasmid was coinjected with low concentrations of Cdc42 plasmid (by itself ineffective), cellular protrusions including microspikes appeared within 90 min after the coinjection, with the peripheral regions undergoing continual retraction and extension over several hours (Fig. 6C, top row). These dynamic and protracted changes resulted in cells displaying extended cytoplasmic tracts 4 h after injection (Fig. 6C, left lower panel). The morphology contrasts sharply with that of control cells injected with plasmids encoding either MRCKα or Cdc42 on their own examined at this time interval.

**DISCUSSION**

We and others have recently reported the isolation of Rho-binding serine/threonine kinases (ROKs) which act downstream of Rho (1, 2, 19, 20, 23, 30, 31). The isolation of another family of ROK-related kinases with Cdc42 and weak Rac1 binding (MRCKs) strengthens the notion that functionally related members of these kinases are adapted to different switches for diverse biological activities. These multidomain kinases show some similarity in domain organization, with coiled-coil α-helix, cysteine-rich, and PH domains, although the exact arrangements differ. They also share substrates. Like ROKα, MRCKα readily phosphorylates MLC-2 predominantly at serine 19. Phosphorylation of this residue has been reported to be essential for the activation of myosin in vitro (6, 22) and its subsequent effect on the actin-myosin contractile apparatus which has been suggested to underlie the formation of stress fibers and focal adhesion complexes (12). With MRCKα, the exact relationship of its kinase activity to its morphological action remains to be established. It is plausible that phosphorylation of myosin(s) is a common feature of these different kinases and that the site of action will determine the appropriate morphological activity, with selectivity being imparted by specific p21-binding domains.

MRCKα has a characteristic cellular localization which is different from that of ROKα. In general, ROKα is distributed evenly in the cytoplasm and concentrated in the cell periphery only upon translocation by transfected RhoA (31). MRCKα is stained in punctate structures in the cytoplasm, with more intense staining at the periphery of transfected cells, particularly at the leading edge and cell-cell junction. This localization may in part be due to nonkinase regulatory domains such as the PH domain, since its deletion resulted in a more even cellular distribution. The PH domain of the Ras exchange factor Sos had been shown to play a role in targeting the protein to the cell periphery and leading edge of motile cells, in response to serum stimulation (11). Similarly, the N-terminal PH domain of pleckstrin is required for its membrane
localization and induction of membrane projections which is regulated by its phosphorylation (33). Although the families of ROKs and MRCKs contain PH domains, these are not identical, and it would be interesting to determine whether and how the different PH domains influence membrane localization.

Not unexpectedly given the similarity in the kinase domain, overexpression of ROKs and MRCKs can result in overlapping morphological activities under certain experimental conditions. Introduction of plasmids for either ROKα or MRCKα led to enhanced formation of stress fibers and focal complexes, which require their kinase activity (Fig. 5 and references 1, 20, and 30). However, while the kinase-dead (dominant-negative) ROKα mutant effected dissolution of stress fibers and focal adhesion complexes, in keeping with ROK’s role downstream of Rho, kinase-dead MRCKα did not affect these Rho-dependen
t structures. This finding strongly indicates that the functional role of MRCKs is different from that of ROKs. Several lines of evidence from the work presented here suggest that MRCKα is associated with Cdc42 functions. First, Cdc42 co-localizes with MRCKα on cotransfection. Second, introduction of the kinase-dead MRCKα blocks the morphological effects of Cdc42 (12). Third, coexpression of MRCKα with limiting concentrations of wild-type Cdc42 (which elicits no effect on its own) promoted the formation of dynamic peripheral structures including microspikes and filopodia. The formation of these structures require Cdc42 mediation (24, 41).

The relationship of MRCK to other the Cdc42-binding kinase PKA clearly warrants further investigation. αPKA disassembles stress fibers and focal adhesion complexes in HELa cells when activated (34). It has been suggested that this disassembly may facilitate (or be necessary for) the formation of the Cdc42-dependent peripheral structures perhaps because of shared components or cytoskeletal needs, reflecting opposing roles of ROK and PKA (32). In cells injected with activated PKA, dissolution of the Rho-mediated structures is followed eventually by massive cell contraction, with long retraction fibers being visible 90 to 120 min after injection (34). In the present study, coexpression of limiting concentrations of Cdc42 with MRCKα led to pronounced microspike activity and restructuring of peripheral portions of the cell, involving continual retraction and protrusion over an extended time (4 h). Rather than the final overall cell contraction observed with PKA, this restructuring resulted in marked expansion of some parts of the cytoplasm. This finding suggests that MRCK and PKA activities may need to be coordinated in normal cells displaying Cdc42-mediated effects. (It is possible that the Cdc42-binding nonkinases such as the Wiskott-Aldrich syndrome protein, n-chimaerin, related to both the regulatory domain of protein kinase C and BCR, the product of the breakpoint cluster region gene, J. Biol. Chem. 265:1256–1257. 4. Chrzansowska-Wodnicka, M., and K. Burridge. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J. Cell Biol. 133:1403–1415. 5. Coso, O. A., M. Chiariello, J.-C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, H. Ishizaki, T. Maekawa, K. Fujisawa, K. Okawa, A. Iwamatsu, and Y. Kamisoyama, H., Y. Araki, and M. Ikebe. 1990. Novel human brain cDNA encoding a 34,000 Mr protein, n-chimaerin, related to both the regulatory domain of protein kinase C and BCR, the product of the breakpoint cluster region gene. J. Biol. Chem. 265:1146–1147. 6. Drechsel, D. N., A. A. Hymas, A. Hall, and M. Glotzer. 1997. A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos. Curr. Biol. 7:12–23. 7. Fu, Y.-H., A. Pizzuti, R. G. Fenwick, Jr., J. King, S. Rajnarayan, P. W. Dunne, J. Dubel, G. A. Nasser, T. Ashizawa, P. de Jong, B. Wiehring, R. Kedersha, M. R. Perriman, F. Epstein, and C. T. Caskey. 1992. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science 255:1256–1257. 8. Grant, J. T., R. Q. Zhong, O. M. McEwen, and S. L. Church. 1995. The role of the PH domain in the signal-dependent membrane targeting of Snap23. EMBO J. 14:3403–1415. 9. Hall, C., M. Monfrins, P. Smith, H. H. Lim, R. Kozma, S. Ahmed, V. Van- niasingham, T. Leung, and L. Lim. 1990. Human nonphosphorylated 20,000 Da myosin regulatory light chain cDNA. Nucleic Acids Res. 18:5892. 10. Hart, M. J., M. G. Callow, B. Souza, and P. Polak. 1996. IQGAP1, a calmodulin binding protein with a rasGAP-related domain, is a potential effector for Cdc42/Hs. EMBO J. 15:2997–3005. 11. Ishizaki, T., M. Maekawa, K. Fujisawa, K. Okawa, A. Iwamatsu, A. Fujita, N. Watanabe, Y. Saito, A. Kakizuka, N. Mori, and S. Narumiya. 1996. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 15:1885–1893. 12. Ishizaki, T., M. Naito, K. Maekawa, N. Watanabe, Y. Saito, and S. Narumiya. 1997. Development of actin polymerization and proliferation by a pathway distinct from Jun kinase. Science 274:1374–1376. 13. Kamisoyama, H., Y. Araki, and M. Ikebe. 1994. Mutagenesis of the phosphorylation site (serine 19) of smooth muscle myosin regulatory light chain and its effects on the properties of myosin. Biochemistry 33:840–847. 14. Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukuta, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 275:1648–1650. 15. Kozma, R., S. Ahmed, A. Best, and L. Lim. 1995. The Ras-related protein...
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