The Myotonic Dystrophy Kinase-related Cdc42-binding Kinase Is Involved in the Regulation of Neurite Outgrowth in PC12 Cells*

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The myotonic dystrophy kinase-related Cdc42-binding kinase (MRCKα) has been implicated in the morphological activities of Cdc42 in nonneural cells. Both MRCKα and the kinase-related Rho-binding kinase (ROKα) are involved in nonmuscle myosin light-chain phosphorylation and associated actin cytoskeleton reorganization. We now show that in PC12 cells, overexpression of the kinase domain of MRCKα and ROKα resulted in retraction of neurites formed on nerve growth factor (NGF) treatment, as observed with RhoA. However, introduction of kinase-dead MRCKα did not result in NGF-independent neurite outgrowth as observed with dominant negative kinase-dead ROKα or the Rho inhibitor C3. Neurite outgrowth induced by NGF or kinase-dead ROKα was inhibited by dominant negative Cdc42NT17, Rac1NT17, and the Src homology 3 domain of c-Crk, indicating the participation of common downstream components. Neurite outgrowth induced by either agent was blocked by kinase-dead MRCKα lacking the p21-binding domain or by a minimal C-terminal regulatory region consisting of the cysteine-rich domain/pleckstrin homology domain plus a region with homology to citron. The latter region alone was an effective blocker of NGF-induced outgrowth. These results suggest that although ROKα is involved in neurite retraction promoted by RhoA, the related MRCKα is conversely involved in neurite outgrowth promoted by Cdc42 and Rac.

PC12 cells have been used as a model system for investigating neuronal differentiation in vitro (1, 2). Their differentiation has been shown to be stimulated by nerve growth factor (NGF) involving the NGF receptor trk, Ras, Raf1, and mitogen-activated protein kinase cascade (2). Recent studies on PC12 cells and other neuronal cell lines have indicated that the Rho subfamily of GTPases are key mediators of cytoskeletal changes necessary for neurite outgrowth and retraction. Cdc42 and Rac1 mediate outgrowth, whereas RhoA is responsible for retraction (3–5). Genetic studies on the Drosophila central nervous system have also implicated significant roles for Cdc42 and Rac1 in dendritic and axonal growth, respectively (6).

We have recently identified myotin kinase-related serine/threonine kinases MRCKα and β (7), which interact with the GTP-bound form of Cdc42 and to a lesser extent that of Rac, which is related to the RhoA-binding kinases variously called ROK, ROCK, or Rho kinase (8–10). The common catalytic domain of all these related kinases phosphorylates nonmuscle myosin light-chain-2 at serine-19 (7, 11), which is believed to play an important role in myosin contractile activity and associated changes in the organization of actin microfilaments in intact cells (12). In adherent cells in culture, ROKα acts downstream of RhoA in inducing stress fiber and focal adhesion formation (13–15), whereas MRCKα plays a role in mediating filopodia formation as a Cdc42 effector (7). GEK, the Drosophila homolog of MRCK, has also been suggested to regulate actin polymerization as a Cdc42 effector (16). In this study, we have microinjected constructs of MRCKα and ROKα and their various mutant forms into PC12 cells and investigated their effects on neurite morphology. We show that although ROKα can cause neurite retraction, MRCKα may conversely participate in neurite outgrowth downstream of Cdc42 and Rac.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—Full-length ROKα1–1379, kinase-dead ROKαK112A, ROKα1–543, full-length MRCKα1–1732, MRCKα1–473, kinase-dead MRCKα-KD, the triple mutant kinase-dead and p21-binding deficient MRCKα-TM (MRCKα1–473K112A/N113K/R119K), Cdc42NT17, Rac1NT17, and RhoAΔ19 were cloned in pcX40 vector containing N-terminal HA or FLAG epitope as described (7, 13). MRCKα-CPC (amino acids 930–1492) was obtained by first subcloning a 1.4-kilobase BglII fragment from full-length MRCKα into pcX40-FLAG vector to generate a construct containing the cysteine-rich domain (CRD) and vicinal PH domain (CRD/PH domains) and subsequently replaced by an Nhel/Dnrl fragment at the 5′ end. MRCKα-C (amino acids 930–1492/1117–1181) was obtained by an EcoRV/NheI in-frame deletion from MRCKα-CPC construct. MRCKα-C containing the citron homology domain alone (amino acids 1395–1492) was obtained by polymerase chain reaction of the MRCKα full-length DNA with primers 5′-CTGGATCCCTCCAT-TCATAAGTACCCATCTAAATGACCAC-3′ and pcX40 reverse primer. The subsequent BamHI/Dnrl fragment was subcloned into pcX40-FLAG vector. The human Crk-SH3 construct encompassing the C-terminal SH3 domain of CrkII (amino acids 102–304) was derived from an expressed sequence tag cDNA sequence (GenBank accession number AA197286). The ACK-SH3 construct was obtained by subcloning a BamHI/BstElI fragment (amino acids 267–498) from an ACK cDNA (17). All subclones were confirmed by sequencing, and the production of the right sized proteins from these constructs was analyzed by appropriate transfection of COS cells.

Cell Culture, Microinjection, Transfection, and Cell Staining of PC12 Cells—PC 12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum (Life Technologies, Inc.) and 10% horse serum (Sigma). For microinjection of nondifferentiated PC12 cells, subconfluent cells plated on collagen-coated coverslips for 48 h were microinjected with different constructs (50 ng/μl) 2–4 h after injection, cells were fixed with 4% paraformaldehyde and stained with the combination of various primary antibodies: anti-HA (12CA5; Roche Molecular Biochemicals) or anti-FLAG, (M2; IBI). Stained cells were analyzed with an MRC 600 confocal imager adapted to a ZEISS Axioplan microscope. For differentiation, 50 ng/ml NGF (human recombi-
nant; Sigma) in serum-free medium was used. After 3 days of NGF treatment, cells were microinjected with different constructs and stained as described previously (13). For PC12 cell transfection, subconfluent cells plated on 20 μg/ml laminin-coated coverslips were transfected with various HA- or FLAG-tagged DNA constructs (1 μg/ml) with LipofectAMINE (Life Technologies, Inc.) according to recommended protocol. 5 h after transfection, cells were exposed to 50 ng/ml NGF in medium containing 2% serum for 48 h before fixing and staining as described above. For evaluation of neurite outgrowth, the total number of cells expressing the HA- or FLAG-tag were counted, and cells with neurite of more than two body lengths were regarded as exhibiting neurite growth. Control cells were either injected or transfected with a pXJ40-GST construct.

COS-7 Cell Transfection and Western Blotting—COS-7 cells grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum were transfected with Crk-SH3, ACK-SH3, MRCKα-CPC, MRCKα-CC, MRCKα-C, and other constructs. 24 h after transfection, cell extracts were obtained with lysate buffer (25 mM HEPES, pH 7.7, 0.15 mM NaCl, 15 mM MgCl₂, 0.2 mM EDTA, 1 mM sodium vanadate, 20 mM β-glycerol phosphate, 5% glycerol, and 0.2% Triton X-100), separated on a SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-HA or anti-FLAG antibodies for expression. Total soluble extracts from rat brain and PC12 were used for Western blotting using anti-MRCKα (monoclonal antibody 1A1, Ref. 13) or rabbit anti-MRCKα raised against the Cdc42-binding domain of MRCKα.

RESULTS

Neurite Retraction in NGF-treated PC12 Cells Is Induced by Overexpression of the Catalytic Domain of Either ROκα or

FIG. 1. The effects of ROκα and MRCKα expression on neurite retraction and outgrowth in PC12 cells. A, ROκα and MRCKα endogenous expression in rat brain and PC12 cells. 100 μg of soluble extract from rat brain and PC12 cells were separated on 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and probed with anti-ROκα and anti-MRCKα antibodies. B, expression of ROκα or MRCKα catalytic domain causes neurite retraction in NGF-differentiated PC12 cells. PXJ40-HA vector encoding ROκα or MRCKα catalytic domain was microinjected into NGF-treated cells. Cells were fixed after 2 h and stained with anti-HA. Arrows indicate the injected cells. Bar = 10 μm in this and other photomicrographs. C, expression of kinase-dead ROκα-KD but not MRCKα-KD induces neurite outgrowth in nondifferentiated PC12 cells. PXJ40-HA vector encoding either ROκα-KD (K112A) or MRCKα-KD (K106A) was microinjected into untreated PC12 cells. Cells were fixed after 2–4 h and stained with anti-HA antibody. C3 was coinjected with a mouse IgG, and cells were fixed after 30 min and stained with anti-mouse secondary antibody.

FIG. 2. Effects of Cdc42N17, RacN17, Crk-SH3, and ACK-SH3 on neurite outgrowth induced either by NGF or ROκα-KD. A, PC12 cells were transfected with a pXJ40-FLAG vector encoding Cdc42N17, RacN17, Crk-SH3 or ACK-SH3. Cells were treated with NGF for 48 h, fixed, and stained with anti-FLAG antibody (left panel). Alternatively, cells were co-injected with the above constructs together with ROκα-KD vector, fixed after 4 h and doubly stained with anti-HA and anti-FLAG antibodies. Only anti-HA staining is shown on the right panel. B, the graphs show the proportion of injected or transfected stained cells with neurite growth of more than 2 body lengths. Control NGF-treated cells were transfected or injected with pXJ40-GST and stained with anti-GST antibody. Results are means ± S.E. from three to four independent transfections. 50 cells were counted for each independent treatment.
MRCKα—Although various isoforms of ROKα and MRCKα are ubiquitously expressed in many tissues and cultured cells, the highest levels are detectable in the brain (7, 13). In PC12 cells, the levels of ROKα and MRCKα are comparable with those in the brain (Fig. 1A).

In nonneuronal cells, expression of either ROKα or MRCKα, or their kinase domains, resulted in increases in stress fibers and focal adhesions (13–15). Here using PC12 cells treated with NGF for 48 h, we find that microinjection of the MRCKα catalytic domain construct resulted in neurite retraction (Fig. 1B) as did that of the ROKα. Similar results were obtained with full-length ROKα or MRCKα (data not shown), which are catalytically active in vitro, phosphorylating similar substrates (7, 13).

Kinase-inactive MRCKα-KD Unlike ROKα-KD Does not Induce Neurite Outgrowth—We have previously shown in HeLa cells and fibroblasts that the kinase-dead ROKα-KD caused rapid losses of stress fibers and focal adhesion, which were not observed with the kinase-dead MRCKα-KD, suggesting distinctive differences in their activity on the actin cytoskeleton (7, 13). In PC12 cells, microinjection of the dominant negative ROKα-KD construct can induce outgrowth in the absence of NGF. This effect was very similar to that observed with C3 toxin injection, which inhibits RhoA by ADP-ribosylation. However, no such effect was observed when MRCKα-KD was expressed in these cells (Fig. 1C). We conclude that inhibition of the catalytic activity of ROKα, but not of MRCKα, can mimic the effects of inhibiting RhoA in PC12 cells in inducing neurite outgrowth. Unlike NGF treatment which gave obvious effects only after 1–2 days, microinjection of either ROKα-KD or C3 toxin produced neurites within hours. These neurites were also more extensively branched than those obtained after NGF treatment (see Fig. 2A and Fig. 3B).

Neurite Outgrowth Induced by NGF and ROKα-KD Employ Common Components—We next investigated whether the action of NGF and of ROKα-KD in inducing neurite outgrowth involved components in common. GTPases such as Cdc42 and Rac1 (3) and the adaptor protein Crk (18, 19) have been shown to be required for neurite outgrowth with their dominant negative mutants being potent inhibitors of outgrowth. As shown in Fig. 2, A and B, transfection of constructs encoding Cdc42(N17), Rac1(N17), and Crk-SH3 but not ACK-SH3 could effectively block NGF-induced neurite outgrowth in PC12 cells. These constructs exerted similar blocking effects on the neurite outgrowth induced by ROKα-KD (Fig. 2, A and B). These data suggest that neurite outgrowth induced either by NGF treatment or inhibition of the RhoA pathway by ROKα-KD expression involve common downstream components, including Cdc42 and Rac.

Mutants of MRCKα Including a p21-binding and Kinase-deficient Mutant and Truncated C-terminal Fragments Block both NGF- and ROKα-KD-induced Neurite Outgrowth—As in other cells, endogenous MRCKα does not appear to be involved in mediating Rho-type actions in PC12 cells. In nonneuronal cells, there is evidence that MRCKα can potentiate the effects of Cdc42 in promoting peripheral changes (7). To determine whether MRCKα exerted a similar peripheral effect in PC12 cells, they were transfected with various plasmid constructs of MRCKα (Fig. 3A) including MRCKα-TM (which was kinase and p21-binding defective), MRCKα-CPC (containing the cysteine-rich/PH domains as well as a region with marked homology to citron; Refs. 20 and 21), and MRCKα-CC (containing the cysteine-rich domain/citron homology region with the PH domain deleted). The response of these transfected cells to NGF treatment was examined. In separate experiments, these constructs were also microinjected together with ROKα-KD to

![Fig. 3. Mutant forms of MRCK-α block both NGF- and ROKα-induced neurite outgrowth.](http://www.jbc.org/)

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**Fig. 3.** Mutant forms of MRCK-α block both NGF- and ROKα-induced neurite outgrowth. A, a diagrammatic representation of the MRCKα mutants used. CR, cysteine-rich domain; PH, pleckstrin homology domain; GBD, GTPase-binding domain; CH, citron homology domain. B, PC12 cells were transfected with pXJ40-FLAG vector encoding the various mutants of MRCKα (depicted in C) and treated with NGF (left panel). Cells were also coinjected with ROKα-KD vector together with each of the MRCKα constructs (right panel). C, graphical analysis of neurite outgrowth as described in Fig. 2.
assess their effects on ROKα-KD-induced neurite outgrowth.

A substantial attenuation of NGF-induced neurite outgrowth was observed with all three mutants, MRCKα-TM, -CPC, and -CC (Fig. 3, B and C). Strikingly, a similar effect was observed when cells were transfected with the mutant MRCKα-C, which contained only the C-terminal region with homology to citron (bottom photograph). MRCKα-TM and MRCKα-CPC also blocked neurite outgrowth induced by ROKα-KD expression but to a lower extent, whereas MRCKα-CC and MRCKα-C were ineffective in this respect. These results indicate that endogenous MRCKα is involved in promoting neurite outgrowth induced by either NGF or ROKα-KD. Further, they revealed the presence of functional domains in MRCKα including cysteine-rich/PH domains and a regulatory citron homology domain. The inhibitory effects were more prominent with NGF treatment, in which the minimal citron homology domain alone was highly effective. With regard to outgrowth induced by ROKα-KD, the PH domain of MRCKα appears to be crucial. The alignment of the important citron homology domain with homologous sequences in the data base is shown in Fig. 4A. Sequence similarities extended from the newly reported mammalian citron kinase (20, 21), the nck-interacting kinase NIK to the yeast guanine nucleotide exchange factor ROM-2. Interestingly cysteine-rich and PH domains are often found in front of the citron homology domain at the C-terminal end of the proteins.

**DISCUSSION**

Neuronal cells in culture respond differently to various growth factors that regulate their neurite morphology (3–5). Treatment of neuroblastoma cells with serum factors such as lysophosphatidic acid (LPA) subsequently activates ROKα and results in neurite retraction. NGF activates Ras and Rap, which may lead to activation of Cdc42 and Rac1. Cdc42 then activates MRCKα, whose enzymatic activity is required for neurite outgrowth. Inhibition of RhoA or of ROKα can also lead to neurite outgrowth requiring MRCKα action; it is possible that the availability/interactions of Crk may play a role in determining whether outgrowth (Ras/Rap) or retraction (RhoA) of neurites ensues.

**FIG. 4.** A, alignment of a novel citron homology domain from rat MRCKα, a Caenorhabditis elegans MRCK homolog (ceMRCK), mouse citron kinase (21), two other C. elegans citron-like proteins (ceLF59A6 and ceW0288), mouse NIK, and the yeast guanine exchange factor (GEF) ROM-2 from the GenBankTM data base using the DNASTar Clustal method. Identical amino acid residues are shown in black, and highly conserved residues are marked with asterisks. A diagrammatic representation of the various proteins containing the citron homology is also shown for comparison. B, a model for the regulation of neurite outgrowth in PC12 cells. Activation of RhoA in PC12 cells by serum factors such as lysophosphatidic acid (LPA) subsequently activates ROKα and results in neurite retraction. NGF activates Ras and Rap, which may lead to activation of Cdc42 and Rac1. Cdc42 then activates MRCKα, whose enzymatic activity is required for neurite outgrowth. Inhibition of RhoA or of ROKα can also lead to neurite outgrowth requiring MRCKα action; it is possible that the availability/interactions of Crk may play a role in determining whether outgrowth (Ras/Rap) or retraction (RhoA) of neurites ensues.
(Fig. 2, B and C), and RapN17 (data not shown). As the c-Crk mutant was a potent inhibitor in both cases, it is possible that c-Crk (c-Crk-I in PC12 cells) provides a link between the RhoA and the NGF signaling pathways. v-Crk has been recently reported to regulate RhoA activity (27), whereas c-Crk is known to mediate Rap1 effects through the exchange factor C3G believed to be essential for sustaining Ras signaling and PC12 differentiation (28). Furthermore, a direct activation of Rac1 through C-CrkII and DOCK180 has also been documented (29, 30). Blocking of the action of RhoA by C3 or ROKa-KD could allow c-Crk to be available for alternate pathways for which Cdc42 and Rac1 are required (see model in Fig. 4B). RasGAP120 and RhoGAP190 interactions may also be crucial to the cross-talk between these two pathways (31). Whereas NGF-induced neurite outgrowth could be blocked with either PD-98059 or SB-203580 (32), which are specific inhibitors for extra cellular signal-regulated kinases and p38 mitogen-activated protein kinase, respectively, these agents were ineffective in preventing ROKa-KD-induced neurite outgrowth (results not shown). Mitogen-activated protein kinase pathways are therefore not involved in the production of neurites through Rho inhibition, and it is possible that the different types of neurite morphologies in the PC12 cells we observe reflects the involvement of different final pathways. As RhoA inhibition is a crucial step in this process, a major step in growth factor signaling may be in fine tuning of the RhoA pathway.

The neurite outgrowth induced by either ROKa-KD or NGF was effectively blocked by the kinase-dead and p21-binding deficient MRCKa-TM mutant, strongly suggesting that MRCKa is an important component involved in the outgrowth process. Another most striking finding was that a fragment containing just the citron homology region of MRCKa was sufficient for blocking NGF-induced neurite outgrowth. This minimal fragment did not affect ROKa-inhibited outgrowth, whose blocking requires inclusion of the PH domain in the mutants (Fig. 3). The use of these MRCKa mutants thus also reveals further differences between the NGF- and ROKa-KD-induced processes. The meshwork of neurites that emanate immediately on inhibition of Rho activities suggests that the latter is important in eliciting neurite outgrowth and that the cell is already primed to respond rapidly but perhaps in a disorganized fashion. It is possible that for proper neuritic outgrowth initiated by growth factors such as NGF, apart from stimulating Cdc42 and Rac activities, the regulated dampening of Rho activities is required and that the integration of such activities may explain the relatively longer period required for cells to respond to NGF.

We conclude that in PC12 cells, the two myotonic dystrophy kinase-related kinases, MRCKa and ROKa, are involved in regulating neurite morphology but with contrasting roles as depicted in Fig. 4B. ROKa acts downstream of RhoA in inducing neurite retraction and MRCKa acts downstream of Cdc42/Rac1 in promoting neurite outgrowth. Their related catalytic domains allow MRCKa and ROKa to act on similar components of the actomyosin contractile apparatus, whereas their different p21-binding and regulatory domains are probably responsible for specifying their spatial and temporal effects in cells responding to growth factor stimulation. Although the regulatory domains of both kinases include CRD and PH domains, an important difference is the presence of the citron homology region in MRCKa, but absent in ROKa, which appears to be crucially implicated in NGF-induced neurite outgrowth. The citron homology region is also present in a number of signaling molecules in various organisms (Fig. 4A). The role of this region in macromolecular interactions and especially in defining the actions of MRCKa merits further investigation.

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