Small GTPase Tc10 and its homologue RhoT induce N-WASP-mediated long process formation and neurite outgrowth

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Summary

Rho family small GTPases regulate multiple cellular functions through reorganization of the actin cytoskeleton. Among them, Cdc42 and Tc10 induce filopodia or peripheral processes in cultured cells. We have identified a member of the family, designated as RhoT, which is closely related to Tc10. Tc10 was highly expressed in muscular tissues and brain and remarkably induced during differentiation of C2 skeletal muscle cells and neuronal differentiation of PC12 and N1E-115 cells. On the other hand, RhoT was predominantly expressed in heart and uterus and induced during neuronal differentiation of N1E-115 cells. Tc10 exogenously expressed in fibroblasts generated actin-filament-containing peripheral processes longer than the Cdc42-formed filopodia, whereas RhoT produced much longer and thicker processes containing actin filaments. Furthermore, both Tc10 and RhoT induced neurite outgrowth in PC12 and N1E-115 cells, but Cdc42 did not do this by itself. Tc10 and RhoT as well as Cdc42 bound to the N-terminal CRIB-motif-containing portion of N-WASP and activated N-WASP to induce Arp2/3-complex-mediated actin polymerization. The formation of peripheral processes and neurites by Tc10 and RhoT was prevented by the coexpression of dominant-negative mutants of N-WASP. Thus, N-WASP is essential for the process formation and neurite outgrowth induced by Tc10 and RhoT. Neuronal differentiation of PC12 and N1E-115 cells induced by dibutyryl cyclic AMP and by serum starvation, respectively, was prevented by dominant-negative Cdc42, Tc10 and RhoT. Taken together, all these Rho family proteins are required for neuronal differentiation, but they exert their functions differentially in process formation and neurite extension. Consequently, N-WASP activated by these small GTPases mediates neuronal differentiation in addition to its recently identified role in glucose uptake.

Key words: Tc10, RhoT, Cdc42, N-WASP, Neurite outgrowth, Neuronal differentiation

Introduction

Rho family small GTPases are now regarded as central regulators of the actin cytoskeleton and associated cytoarchitectures determining cell morphology, cell migration, cell motility, cytokinesis and cell adhesion (Hall, 1998; Kaibuchi et al., 1999). They are also involved in signaling pathways regulating gene transcription, cell transformation, differentiation, and apoptosis (Hall, 1998; Zohn et al., 1998; Aspenström, 1999a; Aspenström, 1999b; Kjøller and Hall, 1999). The family currently consists of about 14 members and is grouped into six subfamilies: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG), Cdc42 (Cdc42, Tc10), RhoE/Rnd (RhoE, Rnd1, Rnd2), RhoD and RhoH (Zohn et al., 1998; Aspenström, 1999b).

Among them, RhoA, Rac1 and Cdc42 have been studied most intensively. In fibroblasts, activation of RhoA by the extracellular ligand lysophosphatidic acid (LPA) leads to the assembly of contractile actin stress fibers and associated focal adhesions (Ridley and Hall, 1992). By contrast, exogenously expressed constitutively active forms of RhoD, RhoE and Rnd1 disassemble these cytoskeletal structures by antagonizing RhoA (Murphy et al., 1996; Guasch et al., 1998; Nobes et al., 1998; Tsubakimoto et al., 1999). Rac1 activated by platelet-derived growth factor or insulin induces the assembly of an actin filament meshwork to generate membrane ruffles (lamellipodia) and specific adhesion complexes (Ridley et al., 1992; Nobes and Hall, 1995). Cdc42 activated by bradykinin is responsible for the formation of actin-filament-containing microspikes (filopodia) and associated adhesion complexes (Kozma et al., 1995; Nobes and Hall, 1995). In addition, Cdc42 can activate Rac1 and consequently extension of filopodia is accompanied by concerted lamellipodial spreading. Cdc42-related Tc10 produces peripheral extensions longer than the filopodia formed by Cdc42 (Neudauer et al., 1998).

In neuronal cells, these small GTPases also play important roles in neurite extension or growth cone remodeling. *Clostridium botulinum* C3 exoenzyme, which inactivates RhoA by ADP-ribosylating its effector domain, induces neurite

outgrowth in PC12 pheochromocytoma cells and N1E-115 neuroblastoma cells (Nishiki et al., 1990; Kozma et al., 1997). On the other hand, microinjection of constitutively active RhoA or its target protein ROCK/Rho-kinase/ROK in neurite-extending PC12 or N1E-115 cells as well as the treatment of these cells with LPA causes neurite retraction and growth cone collapse (Tigyi and Miledi, 1992; Jalink et al., 1994; Hirose et al., 1998; Katoh et al., 1998). Microinjection of Cdc42 and Rac1 facilitate the formation of filopodia and lamellipodia, respectively, at the growth cones and along neurites of N1E-115 cells (Kozma et al., 1997). The neuronal growth cone guides the extending neurite towards its target by constantly protruding and retracting filopodia and lamellipodia (Heidemann and Buxbaum, 1991; Bentley and O'Connor, 1994). Filopodia appear to serve as sensors in growth cone guidance, whereas lamellipodia are implicated in neurite extension and cellular movement via membrane extension. Dominant-negative Cdc42(T17N) or Rac1(T17N) interferes with the neurite outgrowth induced by C3 exoenzyme or nerve growth factor (NGF), and thus Cdc42 and Rac1 are required for the neurite outgrowth through the formation of filopodia and lamellipodia, respectively, at the growth cone (Kozma et al., 1997; Chen et al., 1999). Dominant-negative mutants of N-WASP, which is a target protein of Cdc42 and plays essential roles in filopodium formation, prevent neurite outgrowth in PC12 and hippocampal neurons (Banzai et al., 2000). Despite their critical roles in neurite outgrowth, neither Cdc42 nor Rac1 is sufficient by itself for activating the signaling pathway leading to the neurite extension.

To examine the differential roles of Cdc42 subfamily members in cellular process formation, we have cloned a Cdc42 subfamily protein, designated as RhoT, which is most closely related to Tc10. Although Cdc42 was ubiquitously expressed in a variety of tissues and cells, Tc10 and RhoT were differentially expressed in particular muscle types and brain and induced during myogenic or neuronal differentiation. A constitutively active mutant of RhoT formed even longer and thicker processes than those formed by Tc10 in fibroblasts. Remarkably, in neuronal cells both Tc10 and RhoT generated neurites, whereas Cdc42 formed mere filopodia. Tc10 and RhoT as well as Cdc42 bound to and activated N-WASP, and both the process and neurite formation induced by Tc10 and RhoT were mediated by N-WASP. Thus, although these three proteins share N-WASP as a common target protein, they are likely to exert distinct functions in process and neurite formation.

Materials and Methods

Cell culture

Mouse C2 skeletal muscle cells (Yaffe and Saxel, 1977) were cultured as described previously (Endo, 1992). The proliferating myoblasts were maintained at 37°C in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum (FBS) (growth medium). To induce terminal differentiation, ~2×10^5 cells (~20% confluency) were plated in the growth medium on a 100 mm dish and maintained for 16-24 hours, and then the medium was replaced with DME medium supplemented with 5% horse serum (HS) (differentiation medium). Myotubes developed extensively by 96 hours after the shift to the differentiation medium. Mouse Balb/3T3 (Aaronson and Todaro, 1968) and C3H/10T1/2 (10T1/2) (Reznikoff et al., 1973) fibroblasts were cultured in the growth medium. Rat PC12 pheochromocytoma cells (Greene and Tischler, 1976) were maintained in DME medium containing 10% FBS and 5% HS. To induce differentiation, the medium was replaced with DME medium supplemented with 50 ng/ml NGF (2.5 S, Promega) or with 10% FBS, 5% HS and 0.5 mM dibutyryl cyclic AMP (dbcAMP) (Sigma). Mouse N1E-115 neuroblastoma cells (Amano et al., 1972) were maintained in the growth medium. To induce differentiation, the cells were shifted to DME medium containing 0.5% FBS or the growth medium containing 2% dimethyl sulfoxide (DMSO).

cDNA cloning and sequence analyses

Cytoplasmic RNA was prepared from mouse C2 myotubes as described previously (Endo and Nadal-Ginard, 1987), and poly(A)$^+$ RNA was isolated using Oligotex-dT30 Super (Roche). The single-stranded cDNA pool was synthesized with SuperScript II RNase H(–) reverse transcriptase (Invitrogen) from 2 μg of the template poly(A)$^+$ RNA primed with an oligo(dT) primer. Mouse *Tc10* cDNA fragment was cloned from the cDNA pool by reverse transcription (RT)-PCR using a sense (GTCTTCGACCACTACGCAGTCA) and an antisense (GCTATGATAGCCTCATCAAAAAC) primer derived from the human *Tc10* cDNA sequence (Drivas et al., 1990) (DDBJ/EMBL/GenBank accession number M31470). The amplification reaction was carried out on Zymoreactor II (Atto) with Taq DNA polymerase (Qiagen). *RhoT* cDNA fragment was cloned similarly with a sense (GTGCCTTATGTGCTCATCGG) and an antisense (CTGAATGTGACTCTGCATTC) primer derived from a mouse expressed sequence tag (EST) clone (accession number AA920345). The C2 myotube cDNA library constructed in λZAPII (Matsumoto et al., 1997) was screened with these cDNA fragments labeled with [α-^{32}P]dCTP (>111 TBq/mmol, ICN Biomedicals) by using the BcaBEST labeling kit (Takara Shuzo). A 4.00 kb and a 1.85 kb cDNAs including the entire coding regions of Tc10 and RhoT, respectively, were cloned. pBluescript SK(–) phagemids containing cloned cDNAs were obtained by in vivo excision. The nucleotide sequence of the cDNAs was determined with LI-COR 4000 automated DNA sequencing system using a SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies). The nucleotide and amino-acid sequences were analyzed with GENETYX-Mac softwares (Ver. 10.1, Software Development Co.).

Northern blotting and quantitative RT-PCR

Cytoplasmic RNAs of cultured cells were prepared as described previously (Endo and Nadal-Ginard, 1987). Total RNAs of mouse tissues were prepared according to Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Northern blotting was carried out as stated elsewhere (Endo and Nadal-Ginard, 1987). Quantitative RT-PCR was performed as described previously (Kadota et al., 2000). The amplification reaction was conducted according to a step program (at 95°C for 60 seconds, at 58°C for 15 seconds and at 72°C for 60 seconds). The primers used for RhoT amplification were the same as those used for the cloning. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control were as previously described (Kadota et al., 2000). After 50 and 17 cycles of amplification, respectively, these products were in the linear range. The PCR produsts were analyzed by agarose gel electrophoresis.

Epitope-tagging, EGFP-tagging and transfection

Point mutations to generate the constitutively active mutants of Tc10(G18V) and RhoT(G30V) and the dominant-negative mutants of Tc10(T23K) and RhoT(T35N) were introduced in the cDNAs using a Transformer site-directed mutagenesis kit (Clontech Laboratories, Inc.). Coding sequences of the wild-type (wt) and the mutated proteins were fused in-frame to the N-terminal Myc-tag in pEF-BOS/Myc vector. They were also ligated to pEGFP-C1 vector (Clontech). These

recombinant plasmids were transfected to the cultured cells grown on glass coverslips by the calcium-phosphate-mediated method as described previously (Endo et al., 1996). The transiently transfected cells were processed for immunofluorescence microscopy (Endo and Nadal-Ginard, 1998). The fixed and permeabilized cells were incubated with the monoclonal antibody (mAb) Myc1-9E10 recognizing the Myc-tag (Evan et al., 1985) (American Type Culture Collection) and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (affinity-purified, Cappel). To detect actin filaments, rhodamine-phalloidin (Molecular Probes, Inc.) was included in the secondary antibody. The specimens were observed with a Zeiss Axioskop microscope.

Yeast two-hybrid interaction assay

The cDNAs encoding the wt, constitutively active and dominant-negative mutants of Cdc42, Tc10, and RhoT were ligated to the Gal4 DNA-binding domain of the pGBT9 vector (Clontech). A cDNA fragment encoding the N-terminal portion (amino acids 1-275) of N-WASP (Miki et al., 1996; Miki et al., 1998) was fused to the Gal4 activation domain of pACT2 vector (Clontech). The yeast strain Y190 was sequentially transformed with the bait and prey plasmids. Double transformants were selected on plates of minimal synthetic dropout medium lacking leucine and tryptophan (SD/–Leu/–Trp). The activation of *lacZ* reporter gene was analyzed using a β-galactosidase colony-lift filter assay.

Pull-down assay

Coding sequences of the wt small GTPases were ligated in-frame to glutathione *S*-transferase (GST)-tag of pGEX-2T vector (Amersham Biosciences). The GST-tagged recombinant proteins were expressed in *E. coli* strain XL1-Blue and affinity-purified with glutathione-Sepharose 4B (Amersham Biosciences). The cDNA encoding full-length N-WASP was fused in-frame to the hemagglutinin $(HA)_3$-tag of pEF-BOS/HA vector. This recombinant plasmid was transfected into Balb/3T3 cells. 24 hours after the transfection, the cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% Na deoxycholate and 0.1% SDS) containing 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 4 mM $MgCl_2$ and then centrifuged at 16,000 ***g*** for 15 minutes. The supernatant was then recovered. The prepurified GST-tagged small GTPases were loaded with either 1 mM GTPγS or GDP and reapplied to glutathione-Sepharose 4B. The cell lysate was applied to the small GTPase-coupled resin and extensively washed with RIPA buffer, and binding proteins were eluted with 5 mM glutathione in 50 mM Tris-HCl (pH 8.0). The eluted proteins were subjected to SDS-PAGE, and then HA-tagged N-WASP was detected by immunoblotting with anti-HA-tag polyclonal antibody (pAb) (MBL), horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) as a secondary antibody, and Renaissance western blot chemiluminescence reagent Plus (NEN Life Science Products).

Actin polymerization assay

Actin, His-tagged N-WASP, GST-tagged VCA fragment of N-WASP and Arp2/3 complex were prepared as described previously (Rohatgi et al., 1999; Fukuoka et al., 2001). The GST-tagged recombinant wt small GTPases were expressed by using pGEX-6P vectors (Amersham Biosciences), and the GST-tag was removed by treating with PreScission protease (Amersham Biosciences). Actin polymerization was analyzed by monitoring the change in fluorescence intensity of pyrene-labeled actin as described previously (Rohatgi et al., 1999; Fukuoka et al., 2001). The small GTPases were mixed with 10 times the amount of GTPγS and incubated for 10 minutes at 30°C, and the reaction was stopped by adding 10 mM $MgCl_2$. Polymerization reaction mixtures contained 2 μM unlabeled actin, 0.2 μM pyrene-labeled actin, 0.2 mM ATP and appropriate proteins (100 nM His-N-WASP, 200 nM GST-VCA, 60 nM Arp2/3 complex and 400 nM small GTPases) in 80 μl of X buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA and 1 mM DTT) and were preincubated for 5 minutes. The reaction was started by adding the mixture of actin and pyrene-actin to the preincubated protein mixtures, and fluorescence change was measured at 407 nm with excitation at 365 nm in the spectrofluorometer FB-777W (JASCO). The kinetic analyses were performed with software provided by the manufacturer.

Results

RhoT is a member of the Cdc42 subfamily

As the mouse *Tc10* cDNA sequence with the complete coding sequence was not available when we began our study, we applied RT-PCR to clone its cDNA from the mouse C2 skeletal muscle myotube cDNA pool by using the primers derived from the human *Tc10* cDNA sequence (Drivas et al., 1990). Then we screened the C2 myotube cDNA library with the PCR product and cloned a 3996 bp cDNA (accession number AB060650). This mouse *Tc10* cDNA contains the complete coding sequence encoding a 205 amino-acid protein with a calculated M_r of 22,659 (Fig. 1A). This sequence and mouse EST databases indicate that mouse Tc10 lacks the eight amino acids at the N-terminus seen in the human counterpart. A search in mouse EST databases allowed us to notice a partial cDNA sequence of mouse Tc10-like protein with the accession number AA920345. On the basis of this sequence, we cloned a fragment of this cDNA by RT-PCR from the C2 myotube cDNA pool. Using this PCR product, we screened the C2 myotube cDNA library and obtained a 1846 bp cDNA (accession number AB060651) containing the entire coding sequence encoding a 214 amino-acid protein with a calculated M_r of 23,766 (Fig. 1A). The amino-acid sequence predicted from the nucleotide sequence had 78.6% and 64.6% identity to those of Tc10 and Cdc42, respectively. Since this protein was phylogenetically closely related to Tc10 among the Rho family proteins (Fig. 1B), we refer to this protein as RhoT (T comes from Tc10-homologue). RhoT, Tc10 and Cdc42 constitute the Cdc42 subfamily.

Recently, a novel Rho family protein TCL has been reported (Vignal et al., 2000). Except for an N-terminal deletion of 10 amino acids (corresponding to Arg4-Cys13 of RhoT), mouse TCL was identical to RhoT (Fig. 1C). When the cDNA sequences of *RhoT* and *TCL* were compared with the corresponding mouse genomic sequence of chromosome 12, *RhoT* cDNA sequence exactly matched the genomic exon sequence (Fig. 1C). By contrast, the cDNA sequence deleted in the *TCL* cDNA (corresponding to the Arg4-Cys13 of RhoT) did not represent an intron because it did not coincide with the consensus sequences for an intron. Thus, the deletion in *TCL* is not generated by alternative splicing but seems to be generated either by nucleotide polymorphism or artifactually. Cloning of the *TCL* cDNA by RT-PCR (Vignal et al., 2000) and not by cDNA library screening, by which the *RhoT* cDNA was cloned, may be responsible for the deletion.

RhoT contained the conserved motifs for GTPase activity and GTP/GDP-binding (Fig. 1A). The switch I and II regions undergo considerable conformational change depending on the binding of GTP and GDP (Milburn et al., 1990). The sequence

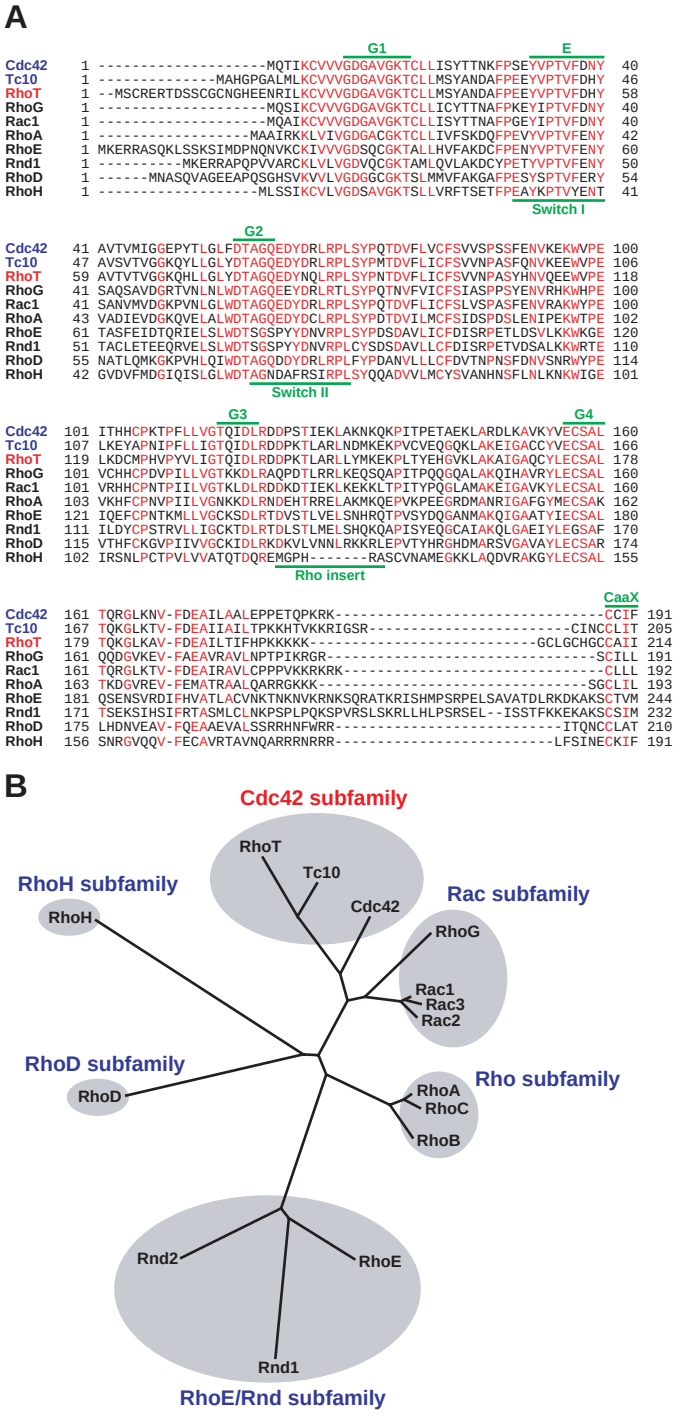

Fig. 1. Structural similarity and phylogenetic relationship of Tc10 and RhoT. (A) Comparison of the amino-acid sequences of mouse Tc10 and RhoT with those of representative Rho family members. The origins of these Rho family proteins are mouse Cdc42 (accession number L78075), human RhoG (X61587), mouse Rac1(X57277), mouse RhoA (AF014371), human RhoE (X95282), human Rnd1 (Y07923), mouse RhoD (D89821) and human RhoH (Z35227). The amino-acid sequences were aligned by the method of Lipman and Pearson (Lipman and Pearson, 1985) and by eye. Amino acids at positions of >50% identity are shown in red. G1-G4, core motifs for GTPase activity and GTP/GDP-binding. E, effector domain. Switch I, switch II, Rho insert regions and CaaX motif are also indicated. (B) Phylogenetic relationship of Rho family proteins. The phylogenetic tree was drawn by using TreeView (Page, 1996). Rho family proteins are subdivided into six subfamilies. RhoT and Tc10 belong to the Cdc42 subfamily. (C) Comparison of the cDNA sequences of mouse *RhoT* (AB060651) and *TCL* (AJ276568) with the corresponding mouse genomic sequence of chromosome 12, which was searched using the Ensembl Genome Browser. Only a portion containing the initiation codon and corresponding amino-acid sequences of RhoT and TCL are shown. Matched nucleotides are marked with asterisks. Gaps are indicated with dashes.

of the switch I region, which almost corresponds to the effector domain, is relatively well conserved among each subfamily. The switch I region sequences were identical in Tc10 and RhoT. Although the sequence of the switch II region is highly conserved among Rho family proteins, RhoT contained uncharged amino acids (NQ) instead of charged amino acids (DR) in this region. The Rho insert region with 13 amino acids is specific for Rho family proteins and has been shown to be involved in the binding of effector proteins and RhoGDI (Freeman et al., 1996; McCallum et al., 1996; Wu et al., 1997). Although the sequences of this region are quite distantly related, those of Tc10 and RhoT were closely related to each other. However, two amino acids ND in this region of Tc10 were replaced by unrelated amino acids LY in that of RhoT. The C-terminal CaaX motif (C, cysteine; a, aliphatic amino acid; X, any amino acid), which is a signal for three types of post-translational modifications, that is, isoprenylation, proteolysis and methylation (Glomset and Farnsworth, 1994; Zhang and Casey, 1996), was conserved in RhoT as well as in the other Rho family proteins. Tc10 as well as H-Ras and RhoB has a CXXC sequence immediately upstream of the CaaX motif. These two Cys residues are palmitoylated and serve as membrane-targeting sites (Hancock et al., 1989; Adamson et al., 1992; Michaelson et al., 2001). Instead of the CXXC, RhoT had a CXXCXXC sequence, which seems to be a site for triple palmitoylation, and another membrane-targeting polybasic region upstream of the CXXCXXC sequence. In addition, RhoT had a long unique N-terminal extension as do RhoD, RhoE and Rnd1.

Tc10 and RhoT are differentially expressed in muscles and brain and induced during myogenic and neuronal differentiation

The expression levels of the Cdc42 subfamily members in tissues and cells were examined by northern blotting (Fig. 2Aa). A 2.2 kb Cdc42 mRNA was ubiquitously present in a variety of mouse tissues examined, whereas a 1.8 kb mRNA

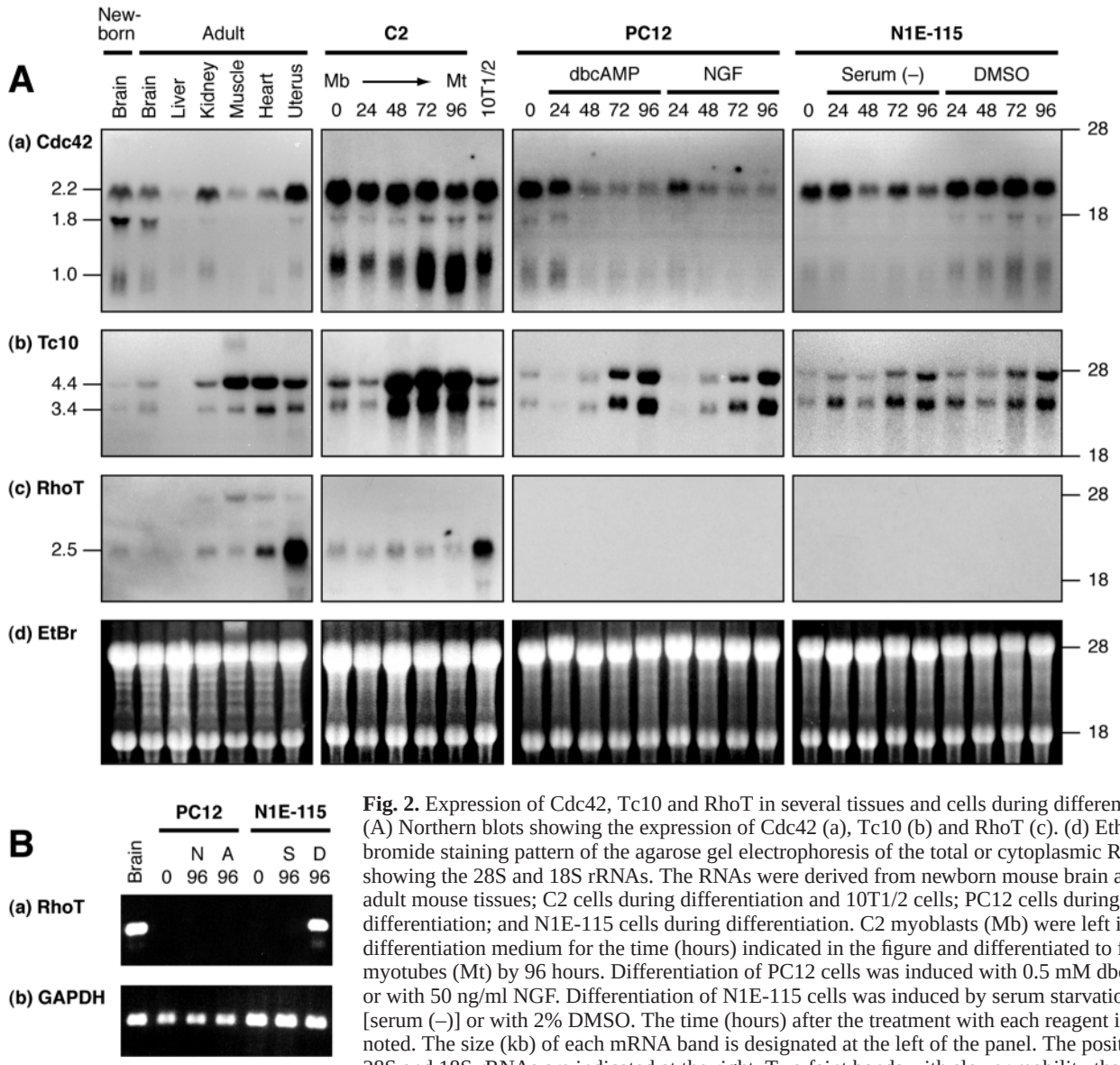

Fig. 2. Expression of Cdc42, Tc10 and RhoT in several tissues and cells during differentiation. (A) Northern blots showing the expression of Cdc42 (a), Tc10 (b) and RhoT (c). (d) Ethidium bromide staining pattern of the agarose gel electrophoresis of the total or cytoplasmic RNAs showing the 28S and 18S rRNAs. The RNAs were derived from newborn mouse brain and adult mouse tissues; C2 cells during differentiation and 10T1/2 cells; PC12 cells during differentiation; and N1E-115 cells during differentiation. C2 myoblasts (Mb) were left in the differentiation medium for the time (hours) indicated in the figure and differentiated to form myotubes (Mt) by 96 hours. Differentiation of PC12 cells was induced with 0.5 mM dbcAMP or with 50 ng/ml NGF. Differentiation of N1E-115 cells was induced by serum starvation [serum (–)] or with 2% DMSO. The time (hours) after the treatment with each reagent is noted. The size (kb) of each mRNA band is designated at the left of the panel. The positions of 28S and 18S rRNAs are indicated at the right. Two faint bands with slower mobility than the 2.5 kb RhoT band in the (c) leftmost panel are unerased Tc10 bands after rehybridization. (B) Quantitative RT-PCR analyses for the expression of RhoT (a) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control (b) in brain and PC12 and N1E-115 cells. PC12 and N1E-115 cells were treated with NGF (N) or dbcAMP (A) and by serum starvation (S) or with DMSO (D) for 96 hours to induce differentiation.

was specifically expressed in the brain. The amount of the 2.2 kb mRNA was almost constant throughout differentiation of C2 skeletal muscle cells and in 10T1/2 fibroblasts. In addition, a 1.0 kb mRNA was detected particularly in these cultured cells. The amount of 2.2 kb mRNA was downregulated during neuronal differentiation of rat pheochromocytoma PC12 cells induced with NGF or dbcAMP and during differentiation of mouse N1E-115 neuroblastoma cells induced by serum starvation. By contrast, it was almost unchanged during differentiation of N1E-115 cells induced with DMSO.

Tc10 mRNAs (4.4 and 3.4 kb) were highly expressed in three types of muscle tissues, that is, leg skeletal muscle, heart (cardiac muscle) and uterus (smooth muscle) (Fig. 2Ab). The mRNAs were also present in brain, and their amounts were higher in adults than in newborns. They existed at a moderate level in undifferentiated C2 myoblasts but were remarkably induced by 48 hours after the induction of differentiation and remained at a high level in terminally differentiated myotubes. Further, the mRNAs were gradually induced during differentiation of PC12 cells stimulated with NGF or with dbcAMP and accumulated at a high level by 96 hours. They were also upregulated in N1E-115 cells according to the progression of differentiation by serum starvation or by DMSO treatment.

By contrast, RhoT mRNA (2.5 kb) was predominantly present in the uterus and heart (Fig. 2Ac). It also existed in skeletal muscle and brain to lesser extents. Its expression level was almost constant during C2 cell differentiation and higher in 10T1/2 fibroblasts than in C2 cells. The mRNA was hardly detected by northern blotting in PC12 and N1E-115 cells regardless of their differentiated state. However, quantitative RT-PCR analyses detected RhoT mRNA in N1E-115 cells,

comparable to the level in the brain, after the differentiation induced with DMSO but not by serum starvation (Fig. 2B). Although the mRNA was not detected in PC12 cells even by the PCR analyses, this was possibly due to the inability of the used PCR primers to anneal to RhoT cDNA because the primers were designed on the basis of the mouse RhoT sequence.

RhoT induces remarkably long and thick processes

Microinjection or transfection of constitutively active Cdc42 to fibroblastic cells results in the reorganization of the actin cytoskeleton and the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995; Dutartre et al., 1996). Transfection of constitutively active Tc10 causes the disassembly of stress fibers and the formation of peripheral extensions longer than those induced by Cdc42 (Neudauer et al., 1998; Murphy et al., 1999). We examined the effects of RhoT on actin cytoskeleton and cell morphology in comparison with those of Cdc42 and Tc10. Transfection of Myc-tagged constitutively active Cdc42(G12V) or Tc10(G18V) to Balb/3T3 and 10T1/2 fibroblasts caused loss of thick stress fibers and induced round cell shape and peripheral processes in both these cell types (Fig. 3A,Ba,c). The processes formed by Tc10 were 10-20 μm long and longer than those by Cdc42 (~10 μm long). They contained actin filaments as detected by rhodamine-phalloidin staining (Fig. 3A,Bb,d). When Myc-tagged constitutively active RhoT(G30V) was expressed in these cells, remarkably long (up to 40 μm) and thick processes containing actin filaments were generated (Fig. 3A,Be,f). The tips of the processes were often swollen, distinct from those formed by Cdc42 or Tc10. Loss of stress fibers and round cell shape were also evident in these cells.

Tc10 and RhoT but not Cdc42 induce neurite outgrowth

Since Tc10 and RhoT caused the formation of long processes and their mRNAs were induced during neuronal differentiation of PC12 or N1E-115 cells, we next examined whether Tc10 and RhoT were responsible for the neurite outgrowth in these cells. If Cdc42(G12V) were transfected into PC12 cells, filopodia were formed but neurites were barely detected (Fig. 4Ab,C). Wild-type (wt) Cdc42 rarely generated even such filopodia (Fig. 4Aa). On the other hand, Cdc42(wt) but not Cdc42(G12V) formed filopodia in N1E-115 cells (Fig. 4Ba,b). Cdc42(G12V) brought about spread or flattened forms of the cells, and neither Cdc42(wt) nor Cdc42(G12V) generated neurites in N1E-115 cells (Fig. 4Ba,b,D).

On the one hand, both Tc10(G18V) and RhoT(G30V) induced neurites in PC12 cells, whereas their wt forms did not (Fig. 4Ac-f,C). Tc10(wt) and RhoT(wt) generated long (>four times longer than the cell body) neurites in N1E-115 cells (Fig. 4Bc-e,D). The neurites induced by RhoT were generally longer than those induced by Tc10. On the other hand, Tc10(G18V) and RhoT(G30V) were less effective in giving rise to the neurites (Fig. 4Bd,f,D). Immunofluorescence microscopy showed that these neurites included microtubules containing tubulin and MAP2 as in the neurites formed in NGF-treated PC12 cells and in serum-starved N1E-115 cells (data not shown). Dominant-negative mutants of Tc10(T23K) and RhoT(T35N) were incapable of forming neurites (data not shown).

Tc10 and RhoT bind to and activate N-WASP to induce Arp2/3-complex-mediated actin polymerization

Cdc42 generates filopodia through binding to the CRIB motif (Burbelo et al., 1995) of its target protein N-WASP, which activates Arp2/3-complex- and profilin-mediated actin polymerization (Miki et al., 1996; Miki et al., 1998; Rohatgi et al., 1999). To address whether Tc10 and RhoT also bound to N-WASP, we exploited the yeast two-hybrid interaction assay. The β-galactosidase colony-lift filter assay showed that both the wt and constitutively active forms of Tc10 and RhoT as well as those of Cdc42 bound to the CRIB-motif-containing N-terminal portion of N-WASP (Fig. 5A). By contrast, their dominant-negative forms and constitutively active RhoA(G14V) as a negative control did not bind to N-WASP.

Next, we assessed the binding of these Cdc42 subfamily proteins to N-WASP by pull-down assay. GST-tagged Cdc42,

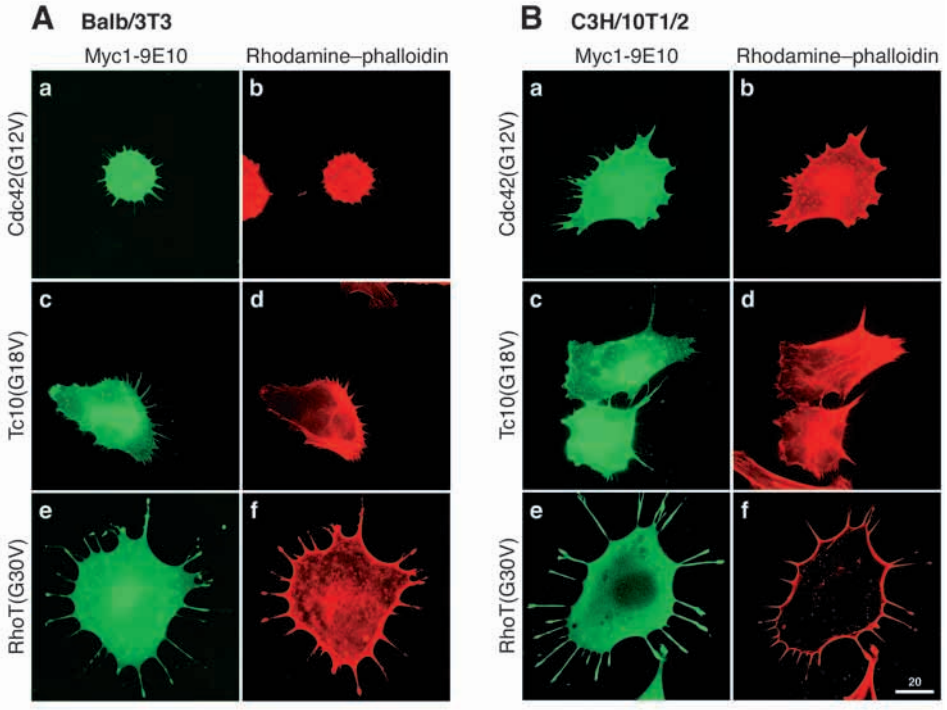

Fig. 3. Distinctive forms of cellular processes generated by Cdc42, Tc10 and RhoT in fibroblasts. Balb/3T3 cells (A) and 10T1/2 cells (B) were transfected with the cDNAs of Cdc42(G12V) (a,b), Tc10(G18V) (c,d) and RhoT(G30V) (e,f) fused to the pEF-BOS/Myc vector. 48 hours after the transfection, the cells were doubly stained with the anti-Myc mAb Myc1-9E10 to detect the Myc-tagged protein (a,c,e) and with rhodamine-phalloidin to reveal the actin filaments (b,d, f). Bar, 20 μm.

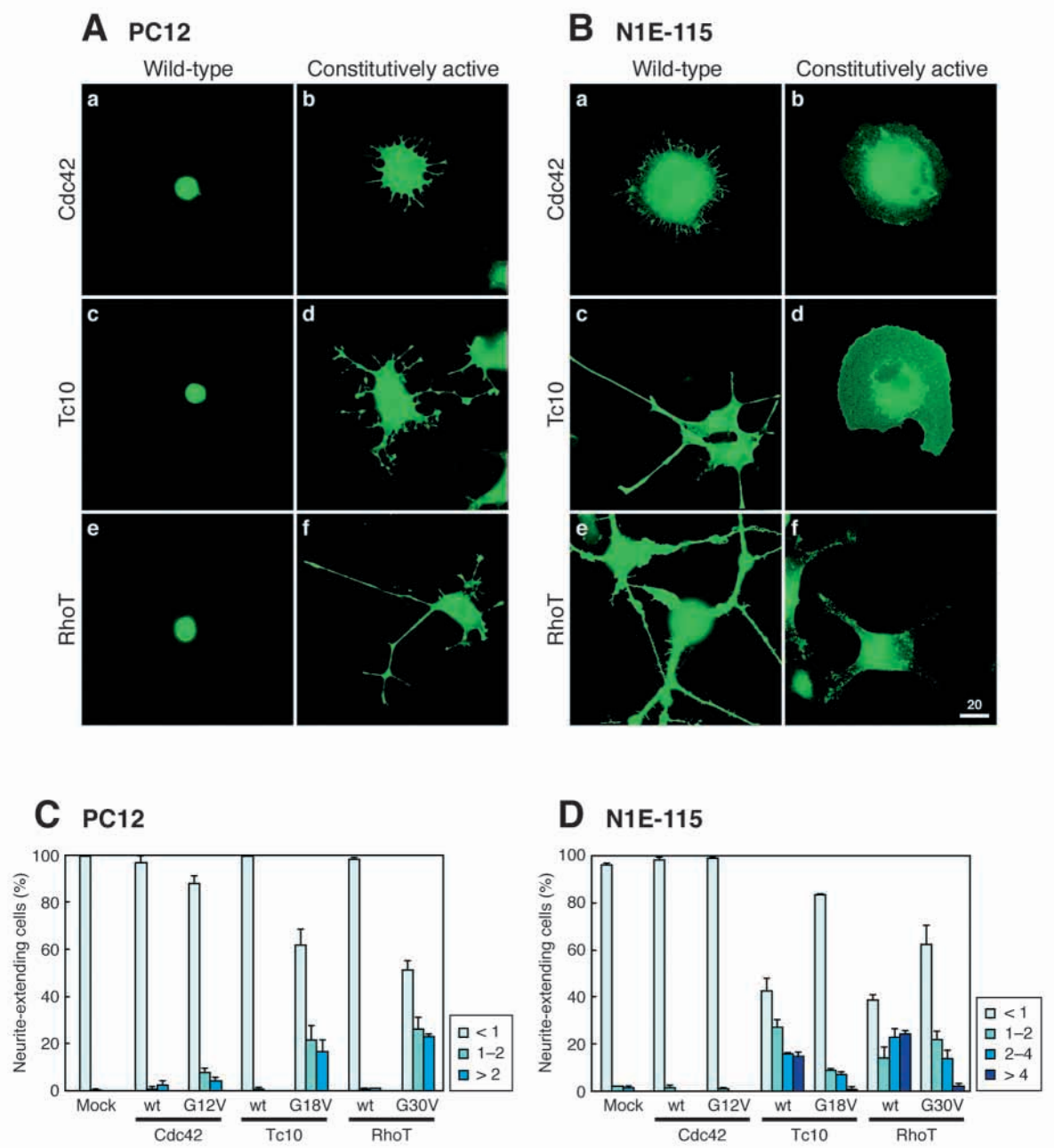

Fig. 4. Generation of neurites by Tc10 or RhoT but not by Cdc42. PC12 cells (A,C) and N1E-115 cells (B,D) were transfected with the cDNAs of wt or constitutively active forms of Cdc42, Tc10 and RhoT fused to pEF-BOS/Myc vector. (A,B) Fluorescent micrographs of the cells expressing the Myc-tagged proteins. Forty-eight hours after transfection, the cells were stained with Myc1-9E10 to detect wt (a,c,e) and constitutively active (b,d,f) forms of Cdc42 (a,b), Tc10 (c,d) and RhoT (e,f). Bar, 20 μm. (C,D) Ratio of the neurite-extending cells. The degree of neurite extension is expressed as a multiple of the cell body diameter as indicated in the legends. More than 100 cells were assessed in each experiment. The values are the means±s.d. of triplicate experiments.

Tc10 and RhoT loaded with GTPγS bound HA-tagged N-WASP expressed in Balb/3T3 cells (Fig. 5B). But the GDP-loaded Cdc42 subfamily proteins as well as GTPγS- or GDP-loaded RhoA were unable to bind N-WASP. Taken together, these results imply that not only Cdc42 but also Tc10 and RhoT bind to N-WASP in a GTP-dependent manner.

To further examine whether the binding of Tc10 and RhoT to N-WASP actually leads to the activation of N-WASP to induce Arp2/3-complex-mediated actin polymerization, we conducted fluorometric actin polymerization assay with pyrene-actin. The VCA (verprolin homology-cofilin homology-acidic domain) fragment of N-WASP with the Arp2/3 complex extremely efficiently activated actin polymerization as described (Rohatgi et al., 1999), whereas N-WASP and Arp2/3 complex did not efficiently activate actin polymerization in the absence of the Cdc42 subfamily proteins (Fig. 5C). Conversely, none of the small GTPases activated actin polymerization without the addition of N-WASP. When

N-WASP and Arp2/3 complex were present, however, both GTPγS-loaded Tc10 and RhoT as well as Cdc42 efficiently activated actin polymerization (Fig. 5C). Thus, Tc10 and RhoT are also concerned with actin polymerization by activating N-WASP as their target protein.

Tc10 and RhoT require N-WASP for process formation and neurite outgrowth

As Tc10 and RhoT bound to N-WASP to induce actin polymerization, it is important to determine whether binding and actin polymerization are essential for the functions of Tc10 and RhoT. The substitution of Asp for His208 (H208D) in the CRIB motif of N-WASP abolishes the binding of Cdc42 to N-WASP (Miki et al., 1998). The cofilin-homology domain of N-WASP in combination with the adjacent acidic domain is involved in the binding of Arp2/3 complex to polymerize actin (Rohatgi et al., 1999; Takenawa and Miki, 2001). The four amino-acid deletion in this domain (Δcof) abrogates the ability to activate Arp2/3 complex (Rohatgi et al., 1999; Banzai et al., 2000). Because both these mutants not only interfere with Cdc42-induced filopodium formation (Miki et al., 1998; Banzai et al., 2000) but also did not affect Rac1-induced lamellipodium formation or RhoA-induced stress fiber formation (data not shown), they actually serve as dominant-negative mutants of N-WASP. Thus, we utilized them to examine the involvement of N-WASP in process formation and neurite outgrowth by Tc10 and RhoT.

When N-WASP(H208D) or N-WASPΔcof was coexpressed with Cdc42(G12V) in Balb/3T3 cells, filopodium formation was prevented (Fig. 6A,Ba,b). These results are consistent with the previous observations using COS-7 cells (Miki et al., 1998). Coexpression of either of these N-WASP mutants with Tc10(G18V) also suppressed the process formation by Tc10 (Fig. 6A,Bc,d). Each of these N-WASP mutants coexpressed with RhoT(G30V) interfered with the RhoT-induced long and thick process formation as well (Fig. 6A,Be,f). Similar results were obtained with 10T1/2 cells (data not shown). These results indicate that N-WASP is required for the Tc10- and RhoT-mediated process formation as well as for the filopodium formation induced by Cdc42.

Coexpression of N-WASP(H208D) or N-WASPΔcof with Cdc42(G12V) in PC12 cells also resulted in the abrogation of filopodium formation (Fig. 7A,Ba,b). Furthermore, Tc10(G18V)-induced neurite outgrowth was hindered by the coexpression of N-WASP(H208D) or N-WASPΔcof (Fig. 7A,Bc,d,C). Similarly, RhoT(G30V)-induced neurite outgrowth was retarded by the coexpression of the dominant-negative mutants of N-WASP (Fig. 7A,Be,f,C). These dominant-negative mutants of N-WASP also interfered with the neurite outgrowth in N1E-115 cells induced by Tc10 or RhoT (data not shown). Thus, N-WASP plays essential roles in the

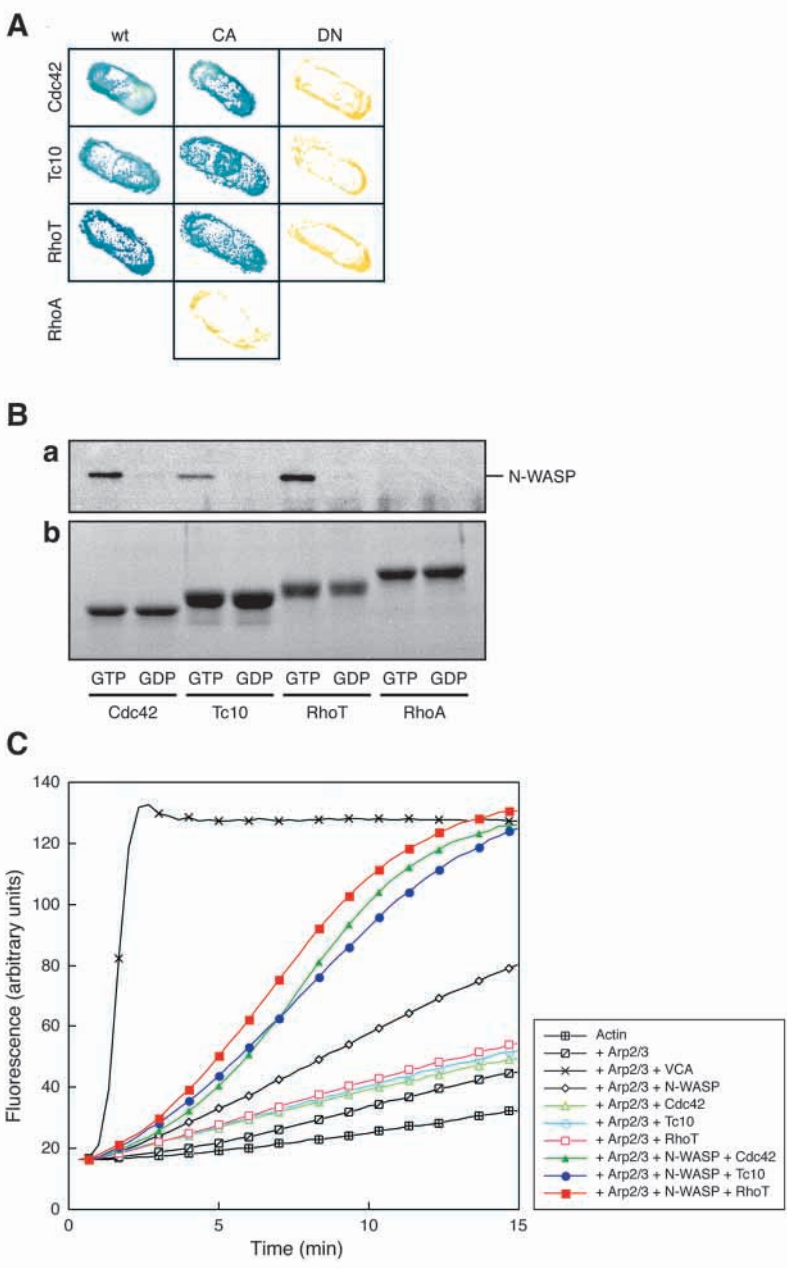

Fig. 5. Binding of Cdc42, Tc10 and RhoT to the CRIB motif of N-WASP leading to the Arp2/3-complex-mediated actin polymerization. (A) Yeast two-hybrid interaction assay. The yeast strain Y190 was transformed with wt, constitutively active or dominant-negative mutants of the small GTPases in bait pGBT9 vector and with the N-terminal portion (amino acids 1-275) of N-WASP containing the CRIB motif in prey pACT2 vector. The interaction was analyzed by β-galactosidase colony-lift filter assay. (B) Pull-down assay. GST-tagged small GTPases loaded with GTPγS or GDP were immobilized to glutathione-Sepharose, and then the lysate from Balb/3T3 cells transfected with HA-tagged N-WASP was applied to the resin. Proteins bound to the small GTPases were eluted, and N-WASP was detected by immunoblotting (a). Used GST-small GTPases were analyzed by SDS-PAGE (b). (C) Fluorometric actin polymerization assay. Protein mixtures contain 60 nM Arp2/3 complex, 100 nM His-N-WASP, 200 nM GST-VCA or 400 nM small GTPases in X buffer. The reaction was started by adding the mixture of 2 μM unlabeled actin and 0.2 μM pyrene-actin to the preincubated protein mixtures, and fluorescence change was measured at 407 nm.

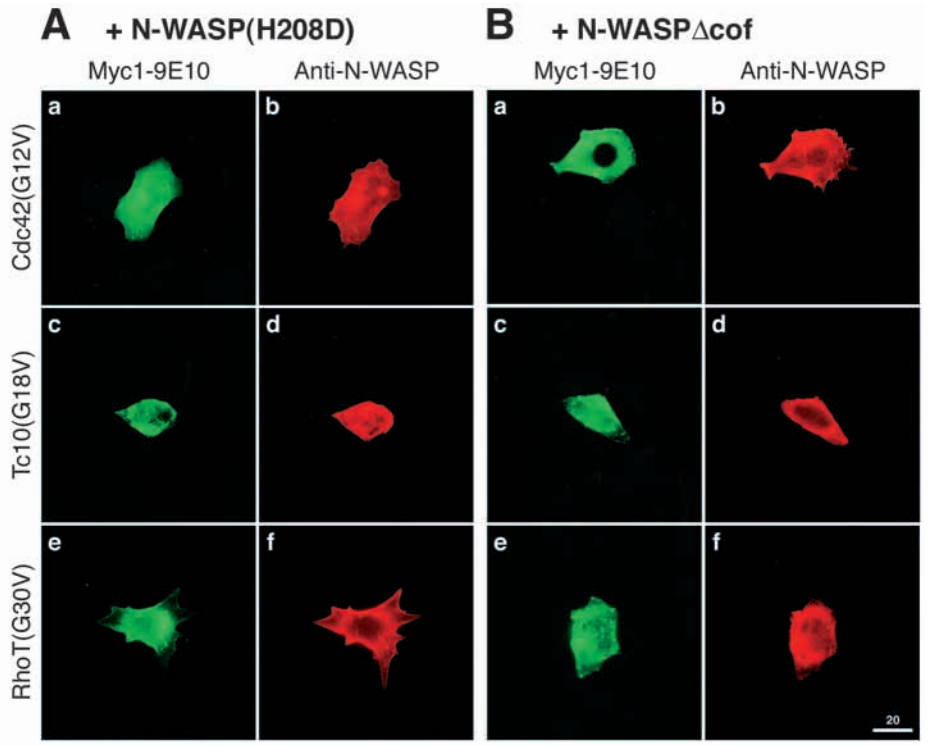

Fig. 6. Prevention of Cdc42-, Tc10- and RhoT-induced process formation by dominant-negative mutants of N-WASP. Balb/3T3 cells were cotransfected with the cDNA of Cdc42(G12V), Tc10(G18V) or RhoT(G30V) in pEF-BOS/Myc vector and that of N-WASP(H208D) (A) or N-WASPΔcof (B) in pcDL-SRα vector. Forty-eight hours after the transfection, the cells were doubly stained with Myc1-9E10 to detect the expression of Cdc42(G12V) (a), Tc10(G18V) (c) and RhoT(G30V) (e) and with anti-N-WASP pAb (b,d, f). Bar, 20 μm.

neurite extension caused by Tc10 or RhoT as well as in the process formation in fibroblasts.

Tc10 and RhoT are essential for neuronal differentiation of PC12 and N1E-115 cells

Next, we investigated whether the Cdc42 subfamily proteins were required for the neuronal differentiation in PC12 and N1E-115 cells represented by neurite extension. PC12 cells were induced to differentiate by the stimulation with dbcAMP (see Fig. 8Aa,c,e,g). Expression of dominant-negative Cdc42(T17N) in dbcAMP-stimulated PC12 cells prevented the neurite outgrowth (Fig. 8Ac,d,C), whereas mock transfection of the empty vector had no effect on the neurite extension (Fig. 8Aa,b). Moreover, the expression of dominant-negative Tc10(T23K) or RhoT(T35N) impeded the neurite outgrowth as well (Fig. 8Ae-h,C).

N1E-115 cells were induced to differentiate by serum starvation (see Fig. 8Ba,c,e,g). The dominant-negative Cdc42(T17N) expressed in serum-starved N1E-115 cells prevented the neurite outgrowth (Fig. 8Bc,d,D), although mock transfection of the empty vector did not affect neurite extension (Fig. 8Ba,b). Furthermore, the expression of dominant-negative Tc10(T23K) or RhoT(T35N) hindered the neurite outgrowth as well (Fig. 8Be-h,D). These results, together with those of transfection of the wt or constitutively active mutants, imply that Tc10 and RhoT are required for neurite extension in both PC12 and N1E-115 cells.

Discussion

We have identified the Rho family protein RhoT. It is closely related to Tc10 and constitutes the Cdc42 subfamily. Although amino-acid sequences of the three Cdc42 subfamily proteins showed similarity throughout the entire length, they had distinct sequences at their N- and C-termini as do the other Rho family proteins. At the N-termini, Tc10 and RhoT were 6 and 18 amino acids longer than Cdc42, respectively. Although roles of the N-terminal sequences of small GTPases have not been well understood, some small GTPases bind particular proteins at their N-termini. For instance, RalA interacts at its N-terminus with phospholipase D1 in collaboration with Arf1 (Jiang et al., 1995; Luo et al., 1998). Rnd1 and RhoE interfere with both Rho- and Rac-mediated reorganization of the actin cytoskeleton. Deletion of the N-terminal six amino acids from Rnd1, however, results in deprivation of the antagonistic effects on the cytoskeleton (Nobes et al., 1998). In addition, Rnd2, which lacks the N-terminal extending sequence present in RhoE and Rnd1, has no observable effects on the cytoskeleton (Nobes et al., 1998). Thus, the long N-terminal sequence of RhoT may be involved in the interaction with some proteins to exert certain cellular functions. Tc10 and RhoT have double and triple palmitoylatable Cys residues, respectively, immediately upstream of the C-terminal CaaX motif, whereas Cdc42 lacks the palmitoylatable Cys residues. Multiple palmitoylation is responsible for the association of small GTPases with the plasma membrane (Michaelson et al., 2001; Watson et al., 2001). In addition, RhoT has a membrane-targeting polybasic region upstream of the triple Cys residues. These differences seems to explain, at least in part, the related but distinctive cellular effects among the Cdc42 subfamily proteins despite their shared binding to the common target protein N-WASP.

Cdc42 was ubiquitously expressed in a variety of tissues and cells. By contrast, Tc10 and RhoT exhibited tissue-specific expression patterns. Tc10 was highly expressed in skeletal muscle, the heart and uterus, and its mRNAs were remarkably induced during C2 skeletal muscle cell differentiation. On the

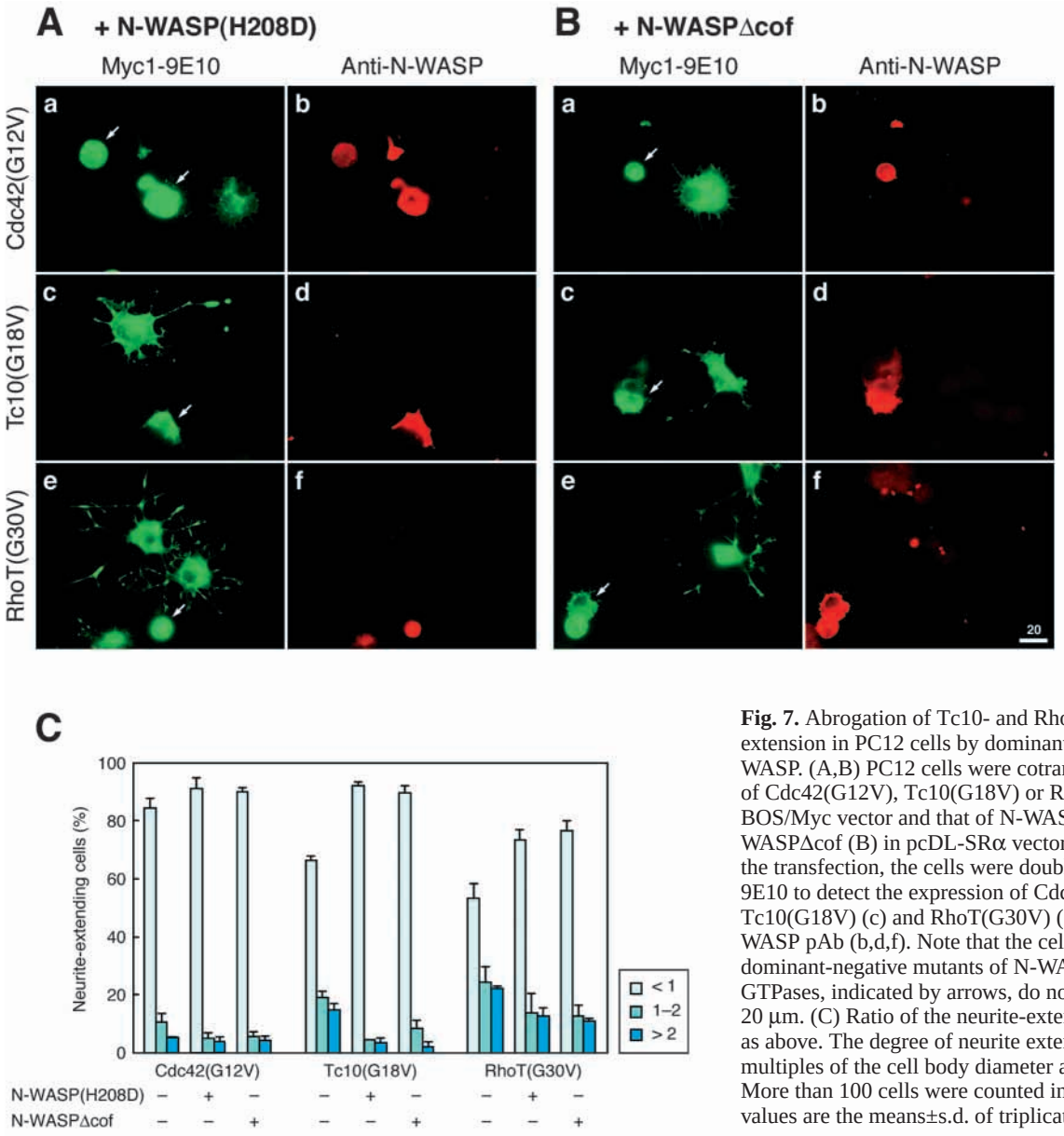

Fig. 7. Abrogation of Tc10- and RhoT-induced neurite extension in PC12 cells by dominant-negative mutants of N-WASP. (A,B) PC12 cells were cotransfected with the cDNA of Cdc42(G12V), Tc10(G18V) or RhoT(G30V) in pEF-BOS/Myc vector and that of N-WASP(H208D) (A) or N-WASPΔcof (B) in pcDL-SRα vector. Forty-eight hours after the transfection, the cells were doubly stained with Myc1-9E10 to detect the expression of Cdc42(G12V) (a), Tc10(G18V) (c) and RhoT(G30V) (e) and with anti-N-WASP pAb (b,d,f). Note that the cells coexpressing the dominant-negative mutants of N-WASP with the small GTPases, indicated by arrows, do not extend neurites. Bar, 20 μm. (C) Ratio of the neurite-extending PC12 cells treated as above. The degree of neurite extension is expressed by multiples of the cell body diameter as indicated in the legend. More than 100 cells were counted in each experiment. The values are the means±s.d. of triplicate experiments.

other hand, RhoT was predominantly expressed in the uterus and heart. These results suggest that Tc10 and RhoT play some roles in these muscular tissues or cells. Indeed, recent studies have shown that Tc10 and its target N-WASP are essential for insulin-stimulated glucose transporter 4 (GLUT4) translocation (Chiang et al., 2001; Jiang et al., 2002), which is required for glucose transport, in skeletal muscle and adipose tissue. GLUT4 is also expressed in heart and smooth muscle (Gould and Holman, 1993) as well as in cultured skeletal muscle cells (Ralston and Ploug, 1996). Accordingly, one of the roles of Tc10 in the muscular tissues and in cultured myocytes is the regulation of glucose transport. Tc10 is located to caveolin-enriched lipid raft microdomains. This location determined by the C-terminal sequence of Tc10, including the double palmitoylatable Cys residues, is indispensible for GLUT4 translocation (Watson et al., 2001). Thus, it is important to examine whether RhoT with triple palmitoylatable Cys residues is also associated with lipid raft microdomains and involved in GLUT4 translocation in smooth muscle and heart.

Tc10 and RhoT were also expressed in the brain, and their mRNAs were induced during differentiation of PC12 or N1E-115 cells. These results seem to imply that Tc10 and RhoT play substantial roles in neuronal differentiation or brain functions. RhoT mRNA was induced by DMSO treatment but not by serum starvation in N1E-115 cells. In this regard, it should be noted that DMSO treatment induces not only neurite extension but also electrophysiological differentiation in N1E-115 cells (Kloog et al., 1983). By contrast, serum starvation also causes neurite outgrowth but does not enhance electrical excitability. This fact may substantiate the roles of RhoT in functional differentiation of neurons. Exogenous expression of the constitutively active Tc10(G18V) in fibroblasts generated peripheral processes containing actin filaments and longer than the filopodia formed by Cdc42(G12V). RhoT(G30V) formed extremely long and thick processes. Notably, the tips of the

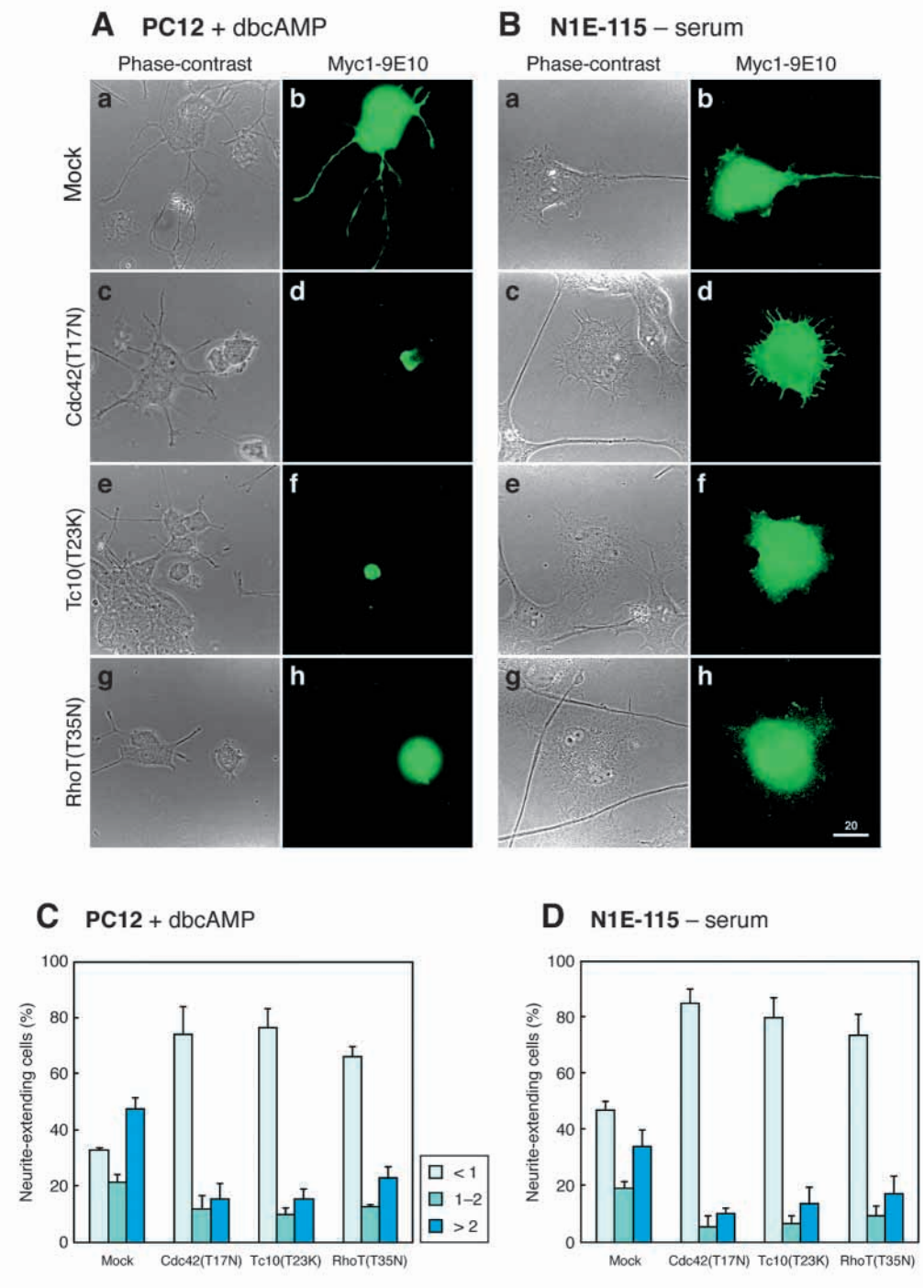

Fig. 8. Suppression of dbcAMP-induced neurite outgrowth in PC12 cells and serum-starvation-induced neurite outgrowth in N1E-115 cells by dominant-negative mutants of Cdc42, Tc10 and RhoT. PC12 and N1E-115 cells were transfected with the cDNA of Cdc42(T17N), Tc10(T23K) or RhoT(T35N) in pEF-BOS/Myc vector or with empty pEGFP-C1 vector (mock). Ten hours after the transfection, PC12 cells were treated with 0.5 mM dbcAMP and the N1E-115 cells were shifted to 0.5% FBS. They were stained with Myc1-9E10 48 hours after the treatment. (A,B) PC12 (A) and N1E-115 (B) cells mock-transfected (a,b) or transfected with Cdc42(T17N) (c,d), Tc10(T23K) (e,f) or RhoT(T35N) (g,h). Shown are phase-contrast (a,c,e,g) and Myc1-9E10-stained fluorescent (b,d,f,h) micrographs. Note that the cells with no expression of these mutants extend neurites. Bar, 20 μm. (C,D) Ratio of the neurite-extending PC12 (C) and N1E-115 (D) cells. The degree of neurite extension is expressed by multiples of the cell body diameter as indicated in the legend. More than 100 cells were assessed in each experiment. The values are the means±s.d. of triplicate experiments.

processes were usually swollen, different from those formed by Cdc42 or Tc10. These long and thick processes with swollen tips are reminiscent of pseudopods of certain types of cultured cells or neurites with growth cones. Recently reported TCL (Vignal et al., 2000) is identical to RhoT except for the N-terminal deletion of 10 amino acids (corresponding to Arg4-Cys13 of RhoT). However, TCL has not been shown to form the peripheral processes or neurites. Instead, EGFP-tagged constitutively active TCL produces actin-rich ruffles on the dorsal cell membrane. The discrepancy between the cellular functions of RhoT and those of TCL is probably due to the artifactual side-effect of the long EGFP-tag for the TCL action because EGFP-tagged RhoT were unable to generate processes any more (data not shown). Alternatively, the inconsistency is ascribed to the N-terminal deletion in TCL because the N-terminal sequence may have crucial functions as discussed above.

Although wt or constitutively active mutants of Cdc42 generate filopodia and successive lamellipodia at the growth cone of N1E-115 cells (Kozma et al., 1997), they scarcely produced neurites in PC12 or N1E-115 cells, consistent with the previous reports (Kozma et al., 1997; Katoh et al., 2000). By contrast, exogenous expression of Tc10 or RhoT induced neurite formation in PC12 and N1E-115 cells. Constitutively

active forms but not wt of these proteins induced neurites in PC12 cells, whereas in N1E-115 cells the wt proteins much more effectively generated neurites than did the constitutively active forms. This may imply that activation of these proteins is essential for the neurite extension in PC12 cells; it may also imply that a regulated activation and inactivation cycle for these proteins favors the neurite extension in N1E-115 cells. RhoT generated neurites longer than those formed by Tc10. These results and those of distinctive process formation in fibroblasts suggest that Tc10 and RhoT possess specific target proteins even if they share several target proteins. Although PC12 and N1E-115 cells are induced to differentiate by different stimulations, the mechanisms for neurite extension with regard to cytoskeletal regulation are likely to be identical or very similar because Tc10 and RhoT induce neurite extension in both these cells.

Expression of each dominant-negative mutant of Tc10 and RhoT prevented the neurite outgrowth in PC12 cells induced by dbcAMP and that of N1E-115 cells induced by serum starvation. Accordingly, neurite extension by Tc10 and RhoT is not merely an artifactual effect of overexpression but indeed a physiological one. Although the exogenous expression of Cdc42 itself did not result in neurite outgrowth, its dominant-negative mutant also interfered with the neurite extension as previously reported (Kozma et al., 1997). This is probably because Cdc42 is essential for filopodium and lamellipodium formation at the growth cone, which is required for neurite extension (Kozma et al., 1997; Gallo and Letourneau, 1998). But formation of these structures on the growth cone may not be sufficient for the induction of neurite outgrowth. Tc10 and RhoT may participate in the elongation of a ‘stalk’ portion of neurites as well as sequential activation of Cdc42 at the growth cone leading to filopodium formation. Indeed, filopodia and lamellipodia were formed at the growth cone of neurites induced by Tc10 or RhoT. As a consequence, exogenously expressed Tc10 or RhoT seems able to generate neurites by itself.

Cdc42 exerts its functions through a variety of effector proteins (Aspenström, 1999a; Aspenström, 1999b). Tc10 shares with Cdc42 some of these target proteins including IQGAP1, PAK, MRCK, MLK, Borg, Par6 and N-WASP (Neudauer et al., 1998; Joberty et al., 1999; Joberty et al., 2000), whereas Tc10 has a specific target protein PIST (Neudauer, 2001). Here we showed that Tc10 and RhoT as well as Cdc42 bound to the CRIB-motif-containing N-terminal portion of N-WASP in a GTP-dependent manner and consequently induced Arp2/3-complex-mediated actin polymerization. Cdc42 generates filopodia through binding to the CRIB motif of N-WASP, which activates Arp2/3-complex-mediated actin polymerization (Miki et al., 1996; Miki et al., 1998; Rohatgi et al., 1999; Takenawa and Miki, 2001). Tc10 and RhoT also utilized N-WASP for the formation of the specific processes distinct from the Cdc42-induced filopodia. This implies that N-WASP mediates the formation of actin filament bundles that are much longer and thicker than those in the filopodia. Furthermore, the neurite outgrowth by Tc10 or RhoT in both PC12 and N1E-115 cells necessitate N-WASP-mediated actin polymerization. Neurite outgrowth induced with NGF or dbcAMP in PC12 cells and that in hippocampal neurons also require active N-WASP (Banzai et al., 2000). In these cells, N-WASP activated by Tc10 or RhoT is likely to be responsible for neurite outgrowth per se and that activated by Cdc42 is responsible for filopodium and lamellipodium formation at the growth cone, because exogenously expressed Tc10 or RhoT but not Cdc42 generates neurites by itself. As stated above, N-WASP activated by Tc10, and possibly by RhoT, is required for GLUT4-mediated glucose uptake in adipocytes and myocytes (Chiang et al., 2001; Jiang et al., 2002). Cdc42 is not involved in this process. Thus, N-WASP activated by Tc10 or RhoT has a variety of physiological functions, which are not mediated by Cdc42. The differential physiological functions among these three members of Cdc42 subfamily seem to be ascribed to their tissue specificity, developmental or differentiation stage specificity and subcellular localization determined by their C-terminal membrane-targeting signals and interaction with target proteins or modulator proteins.

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