Rho GTPases have diverse effects on the organization of the actin filament system

Pontus ASPENSTRÖM[1], Åsa FRANSSON and Jan SARAS[2]
Ludwig Institute for Cancer Research, Biomedical Center, Box 595, S-751 24 Uppsala, Sweden

The Rho GTPases are related to the Ras proto-oncogenes and consist of 22 family members. These proteins have important roles in regulating the organization of the actin filament system, and thereby the morphogenesis of vertebrate cells as well as their ability to migrate. In an effort to compare the effects of all members of the Rho GTPase family, active Rho GTPases were transfected into porcine aortic endothelial cells and the effects on the actin filament system were monitored. Cdc42, TCL (TC10-like), Rac1–Rac3 and RhoG induced the formation of lamellipodia, whereas Cdc42, Rac1 and Rac2 also induced the formation of thick bundles of actin filaments. In contrast, transfection with TC10 or Chp resulted in the formation of focal adhesion-like structures, whereas Wrch-1 induced long and thin filopodia. Transfection with RhoA, RhoB or RhoC induced the assembly of stress fibres, whereas Rnd1–Rnd3 resulted in the loss of stress fibres, but this effect was associated with the formation of actin- and ezrin-containing dorsal microvilli. Cells expressing RhoD and Rif had extremely long and flexible filopodia. None of the RhoBTB or Miro GTPases had any major influence on the organization of the actin filament system; instead, RhoBTB1 and RhoBTB2 were present in vesicular structures, and Miro-1 and Miro-2 were present in mitochondria. Collectively, the data obtained in this study to some extent confirm earlier observations, but also allow the identification of previously undetected roles of the different members of the Rho GTPases.

Key words: actin, Cdc42, GTPase, Rac, Rho.

INTRODUCTION

The Ras proto-oncogene product was discovered more than 20 years ago [1,2]. The revelation that oncogenic mutations in the Ras genes occur frequently in human carcinomas triggered the search for Ras-like genes, and Rho became one of the first gene products to be identified in this effort [1,3]. Since that time, more than 100 Ras-like small GTPases have been identified, which can be classified into five subgroups: Ras, Rab, Arf, Ran and Rho [4,5]. Currently available information from mammalian genome sequencing projects has suggested the presence of a total of 22 Rho family members [6,7]. However, the archetypal triumvirate of Cdc42, Rac1 and RhoA have, so far, received most of the attention, and they each regulate the dynamics of specific actin-dependent cellular processes [8,9]. In addition to being important regulators of the actin filament system, Rho family members participate in signalling pathways that regulate gene transcription, cell cycle entry and cell survival [4,9,10].

Studies on the Rho GTPases have resulted in relatively detailed knowledge with regard to the regulation of these GTP-hydrolysing enzymes: they cycle between inactive GDP-bound and active GTP-bound conformations [8]. This nucleotide-dependent alteration of the conformation allows the enzymes to relay intracellular signals. In the active, GTP-bound, conformation they bind a specific set of signalling molecules, the effector proteins, which in turn transduce the signals from the activated Rho GTPases to induce specific downstream responses [11]. The GDP/GTP cycling of the Rho GTPases needs to be under strict regulation, something which is achieved by GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating proteins) and GDIs (guanine nucleotide dissociation inhibitors). GEFs stimulate the replacement of GDP by GTP, whereas GAPs stimulate the intrinsic GTP hydrolysis of the GTPase [12,13]. GDIs act by blocking GDP dissociation; in resting cells, the Rho GTPases are thought to reside in an inactive complex with GDI proteins, which dissociates upon cell stimulation to allow membrane targeting of the released GTPase [14,15].

The Rho GTPases can be divided into eight subgroups: Cdc42 [Cdc42, TC10, TCL (TC10-like), Chp, Wrch-1], Rac (Rac1–Rac3, RhoG), Rho (RhoA–RhoC), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoD (RhoD and Rif), RhoH/TTF, RhoBTB (RhoBTB1 and RhoBTB 2) and Miro (Miro-1 and Miro-2). RhoG has been suggested to reside in a signalling cascade upstream of Cdc42 and Rac1, as well as to signal in a parallel pathway [16,17]. TC10 was shown to induce filopodia in fibroblasts and nerve elongation in neuronal cells [18,19]. TCL has been shown to induce neurite outgrowth and dorsal veil-like structures in fibroblasts, and a role in adipocyte differentiation has been proposed [20–22]. Chp and Wrch-1 have been implicated in the formation of filopodia in endothelial cells and fibroblasts respectively [23,24]. The Rnd GTPases have been suggested to function in a pathway that antagonizes the Rho-dependent formation of focal adhesions and stress fibres [25,26]. RhoD and Rif also induce filopodia, and RhoD has additional specific roles during endocytosis [27–29]. RhoH/TTF was originally identified as a fusion partner with LAZ3/BCL6 in a non-Hodgkin's lymphoma cell line

Abbreviations used: CRIB domain, Cdc42 Rac interactive binding domain; FBS, foetal bovine serum; GAL4-DB, GAL4 DNA-binding domain; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; HA, haemagglutinin; PAE, porcine aortic endothelial; PAK, p21-activated kinase; PDGF, platelet-derived growth factor; SH3, Src homology 3; TCL, TC10-like; TC10L75 (etc.), TC10 in which residue 75 has been replaced by leucine (etc.); TRITC, tetramethyl rhodamine isothiocyanate; WASP, Wiskott–Aldrich syndrome protein; N-WASP, neuronal WASP.

[1] To whom correspondence should be addressed (e-mail pontus.aspenstrom@LICR.uu.se).
[2] Present address: Department of Medical Sciences, University Hospital, S-751 85 Uppsala, Sweden.

[30,31], and RhoH has furthermore been suggested to function as an antagonist of other Rho GTPases during activation of nuclear factor κB and p38 mitogen-activated protein kinase [32]. Several of the less well characterized Rho GTPases appear to have different GTP-binding and GTP-hydrolysing capacities compared with the classical Rho GTPases. Moreover, some of the recently identified Rho GTPases have other domains in addition to the GTP-binding domains, e.g. Chp and Wrch-1 have N-terminal proline-rich domains which might confer binding of SH3 (Src homology 3) domains [23,24]. The RhoBTB and Miro subfamilies are markedly bigger than the classical small GTPases, and they contain additional domain structures as well as the GTP-binding domains [7,33].

The biological roles of all of these Rho GTPases are currently not clear. In the present work, we have studied the effects of all Rho GTPases on the organization of the actin filament system. To allow side-by-side comparison in the same cell system, constitutively activated forms of all family members were transiently transfected into PAE (porcine aortic endothelial) cells, and the effects on the organization of the actin filament system were monitored. These studies should provide deeper insight into the specific effects on actin organization of the different members of the Rho GTPase family.

EXPERIMENTAL

DNA work and the yeast two-hybrid system

EST (expressed sequence tag) clones encoding human TCL, Wrch-1, Rif and RhoBTB2 were obtained from the U.K. Human Genome Mapping Project Resource Centre (Hinxton, Cambs., U.K.). Fragments containing the coding regions of each of these genes were generated by PCR and subcloned into the pRK5Myc vector. The cDNA of rat Chp was a gift from A. Abo (Onyx Pharmaceuticals, Richmond, CA, U.S.A.); TC10 constructs were from I. Macara (University of Virginia, Charlottesville, VA, U.S.A.); Rac2 constructs were from G. Bokoch (The Scripps Research Institute, La Jolla, CA, U.S.A.); Rac3 constructs were from N. Heisterkamp (Children's Hospital, Los Angeles Research Institute, Los Angeles, CA, U.S.A.); RhoG, RhoH and RhoD were from P. Fort (CNRS-UPR 1086, Montpellier, France); RhoB constructs were from H. Mellor (University of Bristol, Bristol, U.K.); RhoC constructs were obtained from the Guthrie cDNA Resource Center (Guthrie Research Institute, Guthrie, OK, U.S.A.); Rnd1, Rnd2 and Rnd3 were from A. Hall (UCL, London, U.K.); RhoBTB1 (KIAA0740) was obtained from the Kazusa DNA Research Institute, Kazusa, Japan. We are most grateful to all of the above-mentioned scientists for generously sharing these reagents. Constitutively active and dominant negative mutants of the Rho GTPases were generated using the Quickchange protocol (Stratagene).

Deletion mutants of TC10L75 (TC10 in which residue 75 has been replaced by leucine), TCLL79, ChpL89 and Wrch-1L107, lacking the respective CAAX boxes, were generated by PCR and subcloned to allow fusion with the GAL4-DB (GAL4 DNA-binding domain) in the pYTH6 vector and transformed into the yeast strain Y190 [34]. The ability of the GAL4-DB-fused small GTPases to bind to Cdc42 effector proteins fused to the GAL4 activation domain was analysed by transforming the vectors encoding the Cdc42 effectors into Y190 cells expressing the various GAL4-DB–GTPase constructs. The cells were grown on medium lacking histidine and supplemented with 25 mM 3-aminotriazole, as described previously [34].

Antibodies

Mouse monoclonal anti-Myc (9E10), rabbit polyclonal anti-Myc and rabbit polyclonal anti-HA (haemagglutinin) antibodies were from Santa Cruz. Mouse monoclonal anti-vinculin and anti-ezrin antibodies were from Sigma. Mouse monoclonal anti-GM130 antibodies were from BD Transduction Laboratories. Mouse monoclonal anti-HA antibodies (12CA5) were from Roche Diagnostics. FITC- and TRITC (tetramethyl rhodamine isothiocyanate)-conjugated anti-rabbit antibodies were from DAKO. TRITC-conjugated anti-mouse antibodies were from Jackson. Alexa Fluor 488-conjugated anti-rabbit antibodies, Alexa Fluor 350-conjugated phalloidin and LysoTracker Green DND-26 were from Molecular Probes. TRITC-conjugated phalloidin was from Sigma.

Cell cultivation and cell transfection

Cos7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) FBS (foetal bovine serum) and penicillin/streptomycin. PAE cells stably transfected with the human PDGF (platelet-derived growth factor) β-receptor, PAE/PDGFRβ cells (clone β1:3) [35], were cultured in Ham's F12 medium supplemented with 10 % (v/v) FBS and penicillin/streptomycin. All cells were cultured at 37 °C in an atmosphere of 5 % CO_2.

Cos7 cells were transfected using the DEAE-dextran method. PAE/PDGFRβ cells were seeded on coverslips and transiently transfected using lipofectAMINE™ (Invitrogen Life Technologies), employing the protocol provided by the manufacturer. For immunohistochemistry, the coverslips were fixed in 3 % (v/v) paraformaldehyde in PBS for 20 min at 37 °C and washed with PBS. The cells were thereafter permeabilized in 0.2 % (v/v) Triton X-100 in PBS for 5 min, washed again in PBS and incubated in 5 % (v/v) FBS in PBS for 30 min at room temperature. Primary as well as secondary antibodies were diluted in PBS containing 5 % (v/v) FBS. Cells were incubated with primary antibodies followed by secondary antibodies for intervals of 1 h, with a washing step in between. The coverslips were mounted on object slides using Fluoromount-G (Southern Biotechnology Associates). Cells were photographed with a Hamamatsu ORCA CCD digital camera employing the QED Imaging System software using a Zeiss Axioplan2 microscope.

Protein production and GST (glutathione S-transferase) pull-down assays

GST–rhotekin (amino acid residues 1–89), GST–PAK-CRIB [amino acid residues 56–267 of human PAK1B (p21-activated kinase 1B), i.e. the CRIB (Cdc42 Rac interactive binding) domain] and GST–WASP-CRIB [amino acid residues 201–321 of human WASP (Wiskott–Aldrich syndrome protein)] were purified as described previously [36]. Cell lysis and precipitation of Rho GTPases with the GST fusion proteins was performed as described previously [36]. The presence of Rho GTPases in the precipitated material was determined by immunoblotting employing the antibodies described in the legend to Figure 7.

RESULTS

Rho family

The subfamily of Rho GTPases consists of 22 members, which can be subdivided further into eight subclasses (Figure 1, left panel). Most of them belong to the classical or typical category, and they

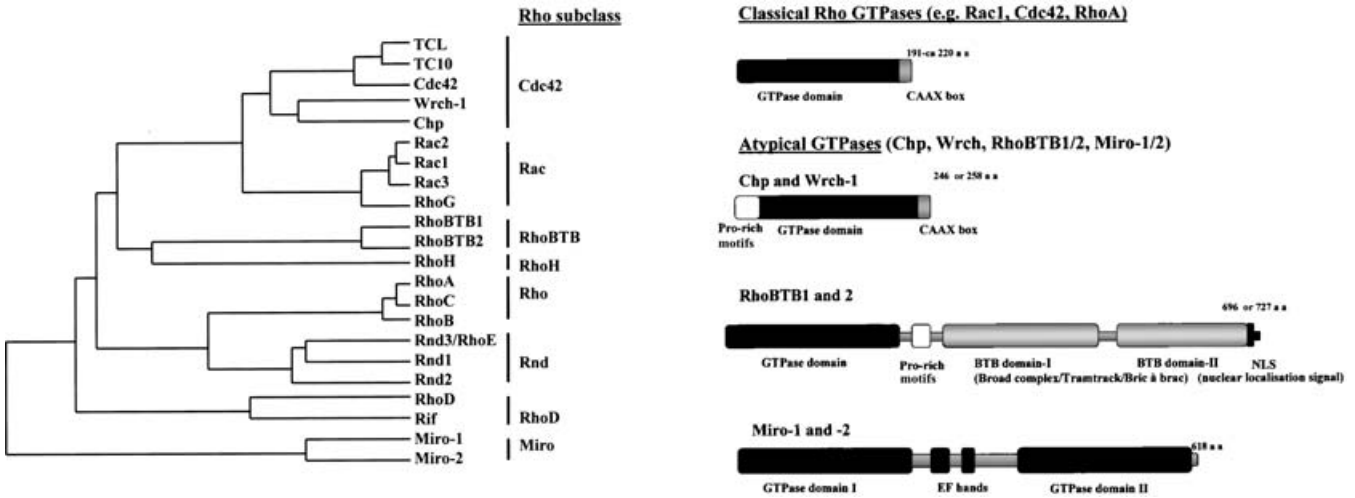


Figure 1 Rho family of small GTPases

Left panel: dendritic tree representation of the Rho GTPases. This representation is based on alignments of the Rho GTPase domains employing the Clustal W algorithm. Right panel: schematic outline of the structure of the Rho GTPases.

are small GTP-binding proteins, each with a C-terminal CAAX motif which undergoes post-translational isoprenylation, most often by a geranyl-geranyl moiety but in some cases by a farnesyl moiety [37,38]. More recently, variations of this theme have become obvious, and three additional types of Rho-type small GTPases have been described. The Chp and Wrch-1 GTPases are similar to Cdc42 and attain a similar domain organization, including C-terminal CAAX motifs (Figure 1, right panel). In addition, these proteins have, in their N-termini, a sequence motif that is rich in proline residues. Interestingly, the N-terminal domain of Wrch-1 was found to confer binding of SH3 domain-containing proteins (J. Saras and P. Aspenström, unpublished work). RhoBTB1 and RhoBTB2 have each a GTPase domain in their N-termini; in addition, they each have a proline-rich motif and two BTB (Broad complex/Tramtrack/Bric à brac) domains (also called POZ domains) [33,39], followed by a possible nuclear localization signal (Figure 1, right panel). Miro has two GTPase domains separated by a linker with EF hands. In this case only the first GTPase domain shares significant similarity with the classical Rho GTPases, whereas the second domain is unrelated to the Rho GTPases [7].

Cdc42 and Rac

PAE/PDGFRβ cells were transiently transfected with plasmids encoding constitutively activated GTPases of the Cdc42 and Rac subclasses. The cells were transfected according to a scheme where-by the cells were fixed 24 h post-transfection, including a 12-h starvation step before fixation. The transfected GTPases were detected by antibodies against the epitope tag on the GTPase, and effects on the organization of the actin filament system were monitored by staining filamentous actin with TRITC-conjugated phalloidin.

Cdc42L61, TCLL79, Rac1L61, Rac2L61, Rac3V12 and RhoGV12 triggered the formation of lamellipodia (Figures 2A and 2B); however, the lamellae in Cdc42L61-expressing cells appeared smaller than in cells expressing Rac GTPases (Figures 2A–2C). Moreover, Cdc42L61, TCLL79, Rac1L61, Rac2L61 and Rac3V12 induced the formation of bundles of actin filaments, a response that was most pronounced in cells expressing Cdc42L61, Rac1L61 and Rac2L61, where the bundles became much thicker than classical stress fibres (Figure 2C). TCLL79-expressing cells had, in addition to lamellipodia and bundles of actin filaments, a number of dotted filamentous actin-containing structures in the lamellae (Figure 2A). These structures are most probably related to focal adhesions, since they also stained positive for the focal adhesion component vinculin (Figure 3A). Cdc42L61, Rac1L61, Rac2L61, Rac3V12 and RhoGV12 induced the formation of focal complexes at the edge of the lamellipodia, visualized with the anti-vinculin antibody (Figures 3A and 3B). In the TC10L75- and ChpL89-expressing cells, the lamellipodia were small and not very prominent (Figure 2A). Interestingly, TC10L75 and ChpL89 appeared to trigger the formation of focal adhesions at the cell periphery, and ChpL89 was present in these focal adhesions. In contrast, Wrch-1L107 induced an extremely spiky phenotype in transfected cells (Figure 2A).

The Cdc42 and Rac GTPases were in most cases localized at the cell periphery, as well as in the perinuclear area, possibly in the proximity of the Golgi apparatus (Figures 2A and 2B). TC10L75 and ChpL89 appeared to be concentrated at focal adhesions (Figure 3A), but the precise localization of Wrch-1L107 was not possible to determine because of the rounded-up phenotype of the transfected cells. Cells expressing Cdc42L61 and Rac1L61 had a number of actin plaques dispersed evenly in the cell body (Figure 2C), a response seen only in cells expressing these two GTPases. Furthermore, in cells transfected with Cdc42L61, Cdc42 frequently formed small ring-like structures or vesicles. These Cdc42-induced vesicles were seen at an even higher incidence in cells expressing wild-type Cdc42 (Figure 2D, arrows). Interestingly, RhoGV12 was localized to the mitochondria (this was also the case for wild-type RhoG), in contrast with all Rho GTPases with the exception of Miro (Figure 2E, arrows).

Rho and Rnd

Ectopic expression of RhoAL63, RhoBL63 or RhoCV14 induced a similar response in the assembly of actin filament bundles. Transfected cells often became small and rounded-up, and several cells also had protrusions, possibly representing retraction fibres

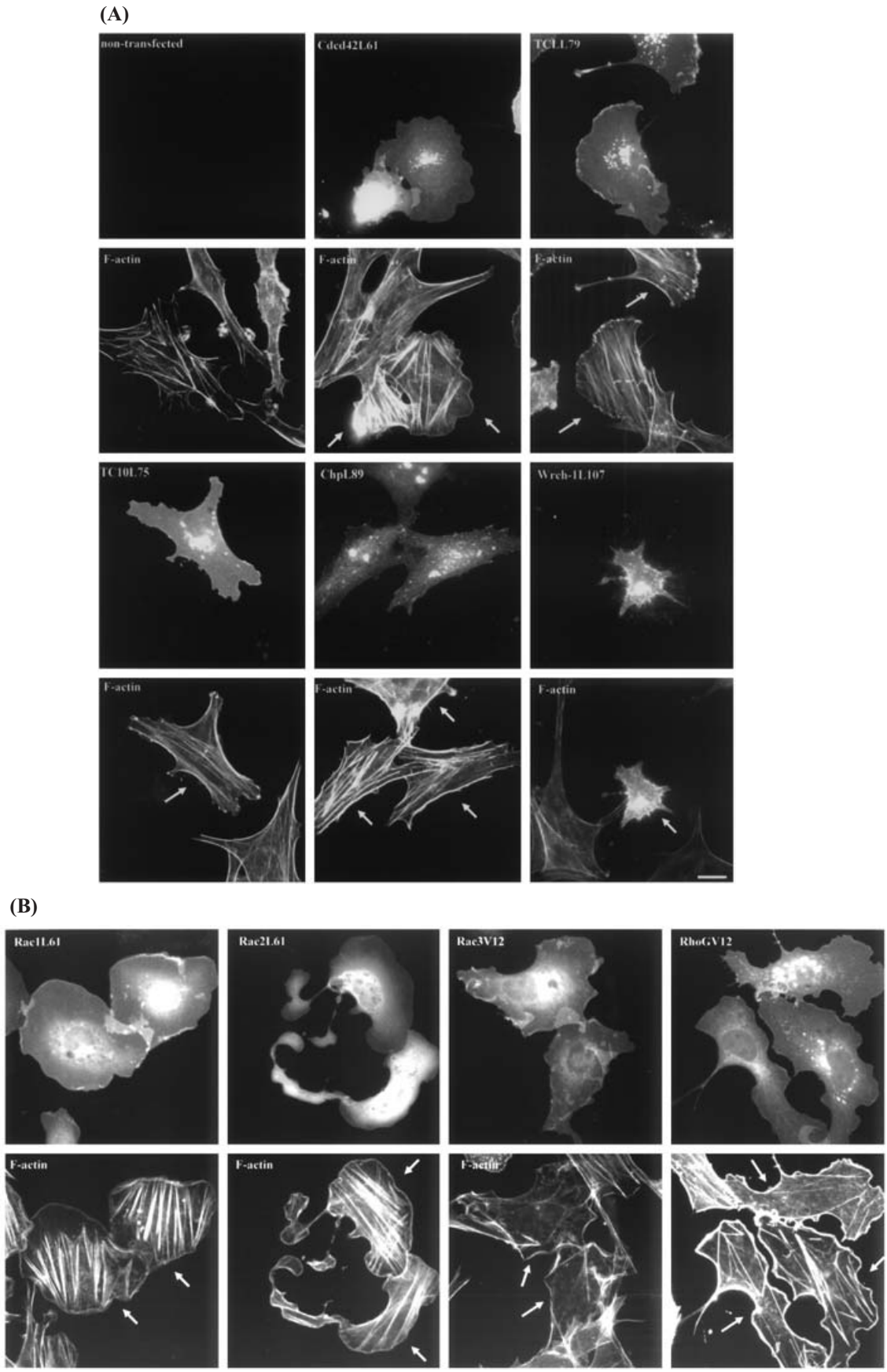


Figure 2 For legend see facing page

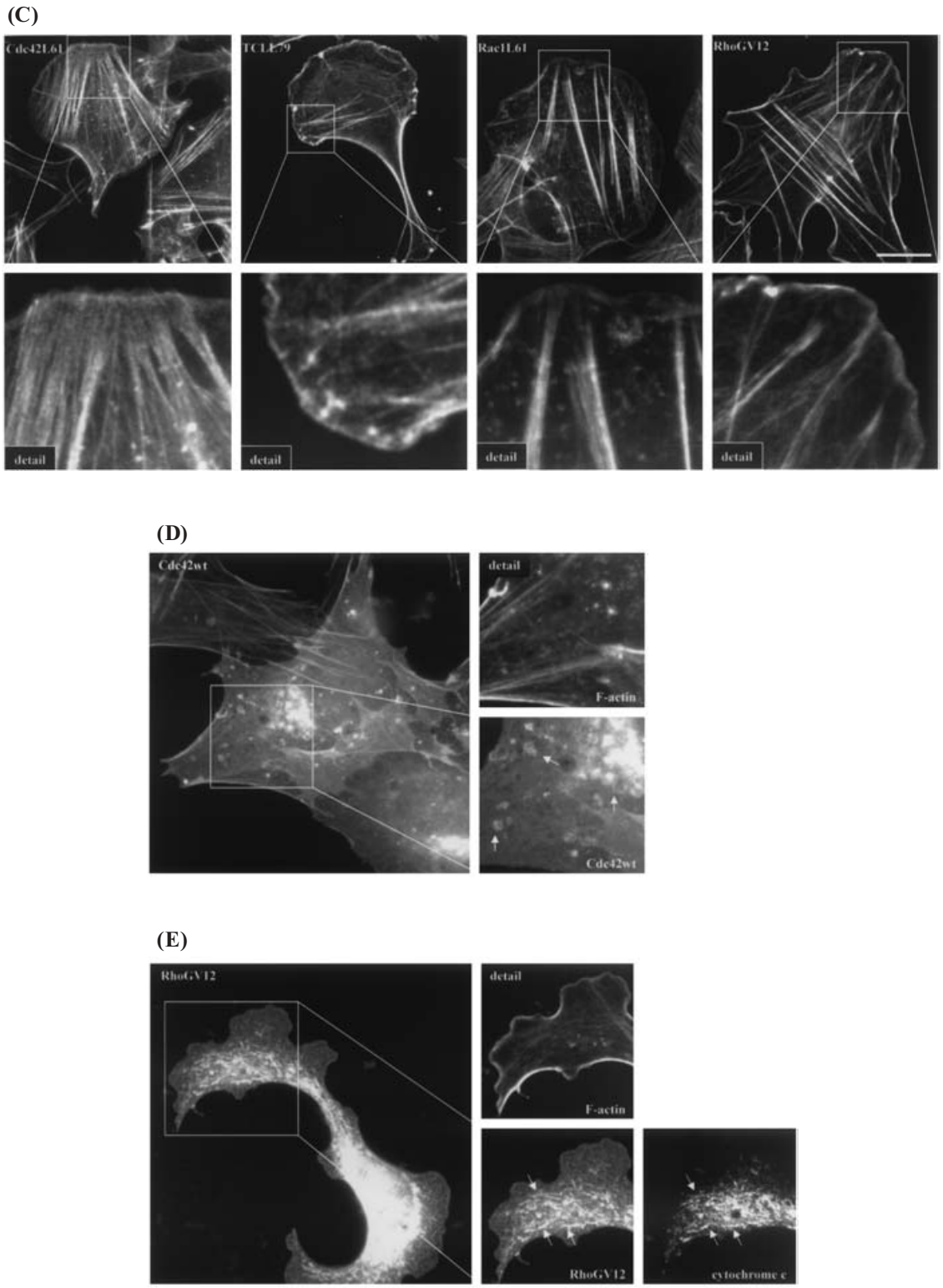


Figure 2 Effects of Cdc42 and Rac subfamily members on the organization of the actin filament system in PAE/PDGFRβ cells

(**A**) The subcellular localization of transiently transfected Cdc42L61, TCLL79 and Wrch-1L107 was visualized with a mouse anti-Myc antibody followed by an FITC-conjugated anti-mouse antibody. TC10L75 and ChpL89 were visualized with a mouse anti-HA antibody followed by an FITC-conjugated anti-mouse antibody. Filamentous actin was visualized with TRITC-conjugated phalloidin. Arrows indicate GTPase-expressing cells. The bar represents 20 μm. (**B**) Transiently transfected Rac1L61, Rac2 L61, Rac3V12 and RhoGV12 were visualized with a mouse anti-Myc antibody followed by an FITC-conjugated anti-mouse antibody. Filamentous actin was visualized with TRITC-conjugated phalloidin. The bar represents 20 μm. (**C**)–(**E**) Transiently transfected Cdc42L61, TCLL79, Rac1L61 and RhoGV12 (**C**), Cdc42wt (wild-type Cdc42) (**D**) and RhoGV12 (**E**) were visualized as described above. (**E**) Mitochondria were visualized with a mouse anti-(cytochrome *c*) antibody followed by a TRITC-conjugated anti-mouse antibody. Arrows indicate the presence of Cdc42 in ring-like structures (**D**) and of RhoG in mitochondria (**E**). The bar represents 20 μm.

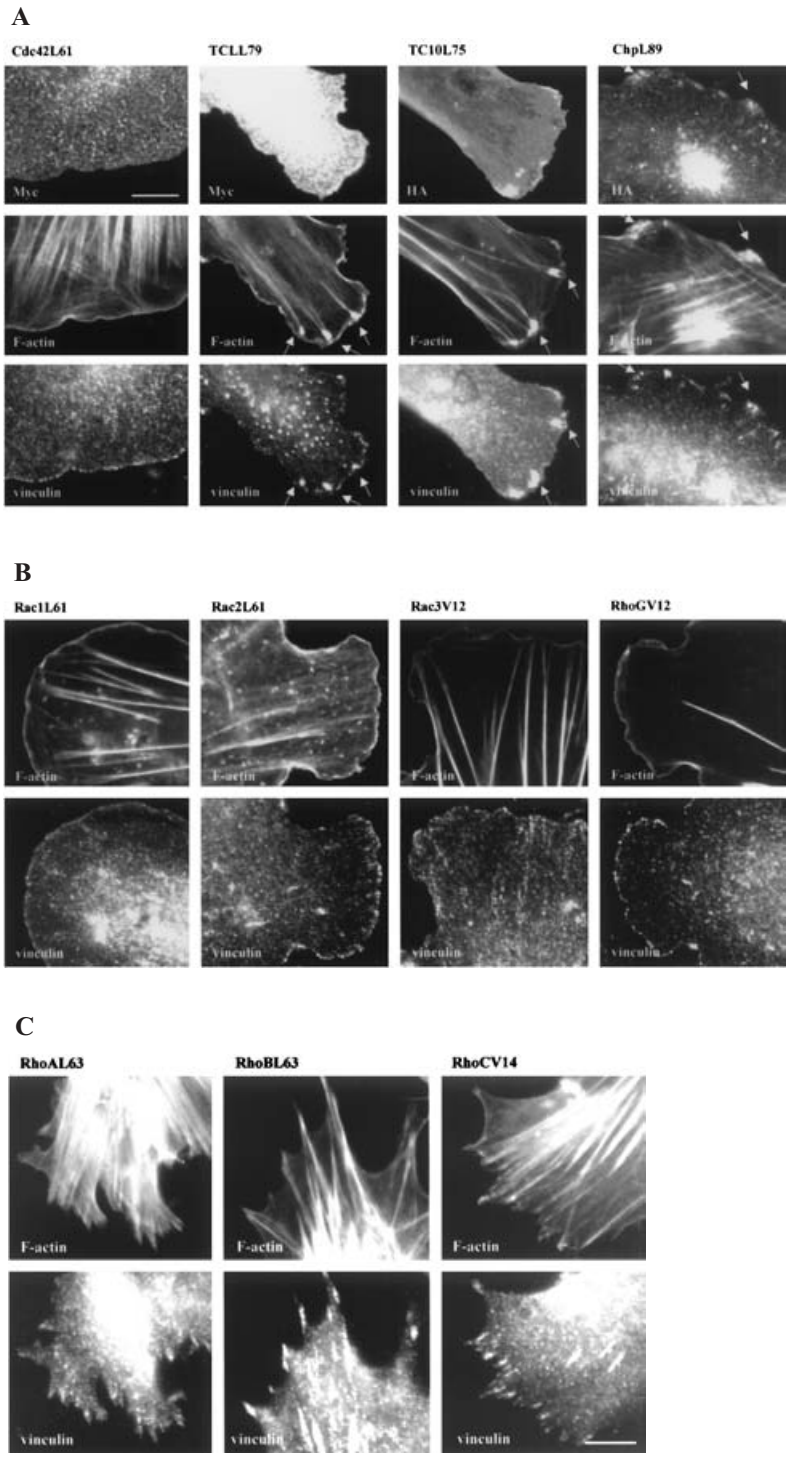


Figure 3 Effects of Cdc42, Rac and Rho on the organization of focal adhesions in PAE/PDGFRβ cells

The organization of focal adhesions was visualized with a mouse anti-vinculin antibody followed by a TRITC-conjugated anti-mouse antibody. Filamentous actin was visualized with Alexa Fluor 350-conjugated phalloidin. (**A**) Cdc42L61, TCLL79 and Wrch-1L107 were visualized with a rabbit anti-Myc antibody followed by an FITC-conjugated anti-rabbit antibody. TC10L75 and ChpL89 were visualized with a rabbit anti-HA antibody followed by an FITC-conjugated anti-rabbit antibody. (**B**) Rac1L61, Rac2L61, Rac3V12 and RhoGV12 were visualized with a rabbit anti-Myc antibody followed by an FITC-conjugated anti-rabbit antibody. (**C**) RhoAL63 and RhoBL63 were visualized with a mouse anti-rabbit antibody followed by an FITC-conjugated anti-rabbit antibody. RhoCV14 was visualized with a rabbit anti-HA antibody followed by an FITC-conjugated anti-rabbit antibody. The bar represents 10 μm.

(Figure 4A). Focal adhesions were prominent in cells expressing the Rho isoforms (Figure 3C). RhoAL63 and RhoCV14 were localized at the plasma membrane; in contrast, RhoBL63 exhibited a spotted localization in transfected cells (Figure 4A). This is consistent with the reported localization of RhoB to late endosomes [40].

Next, PAE/PDGFRβ cells were transfected with Rnd1, Rnd2 or Rnd3. In these experiments the wild-type Rnd proteins were

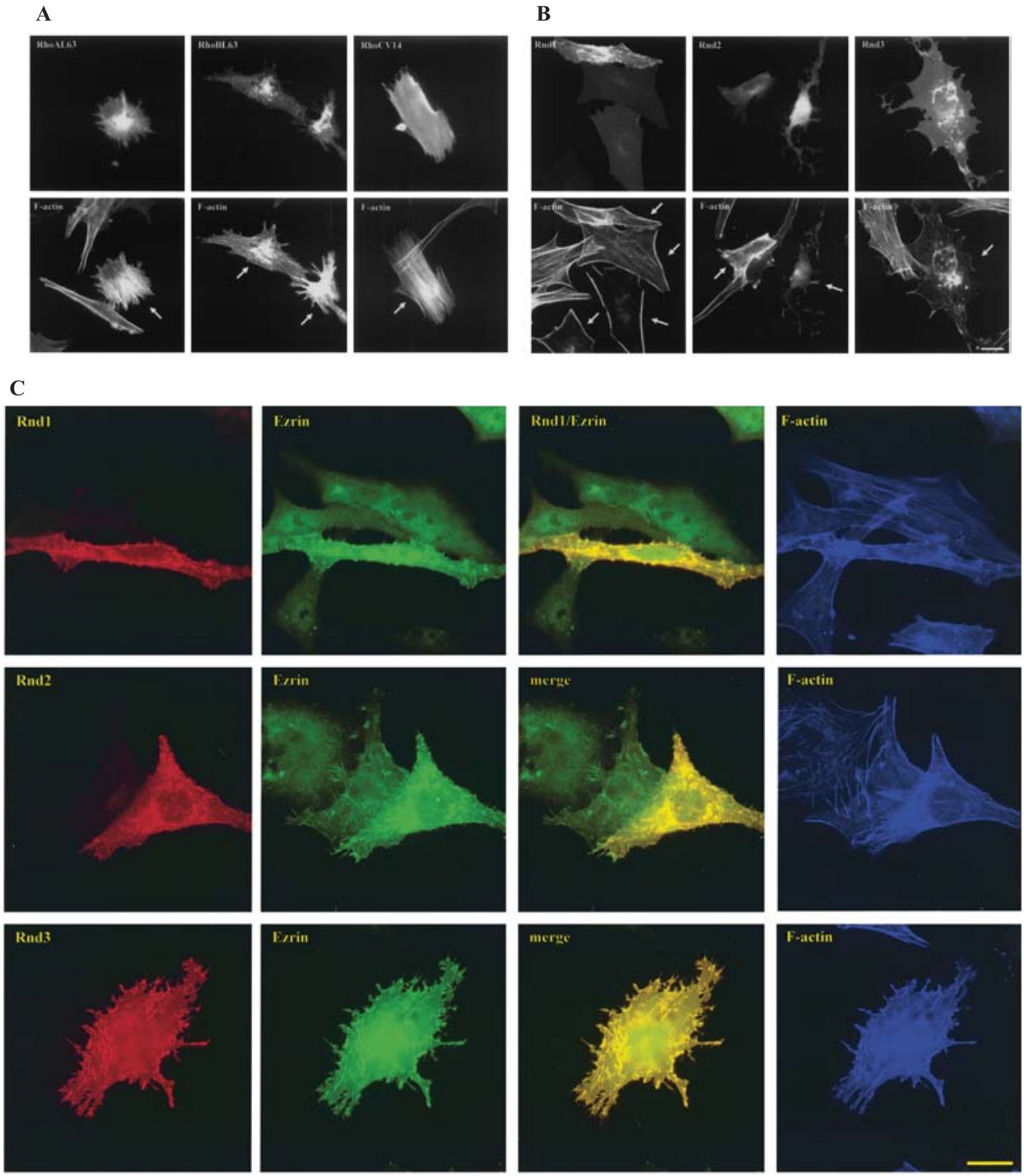


Figure 4 Effects of Rho and Rnd subfamily members on the organization of the actin filament system in PAE/PDGFRβ cells

(**A**) The subcellular localization of transiently transfected RhoAL63 and RhoBL63 was visualized with a mouse anti-Myc antibody followed by an FITC-conjugated anti-mouse antibody. RhoCV14 was visualized with a mouse anti-HA antibody followed by an FITC-conjugated anti-mouse antibody. Filamentous actin was visualized with TRITC-conjugated phalloidin. Arrows indicate GTPase-expressing cells. The bar represents 20 μm. (**B**) The subcellular localization of transiently transfected Rnd1, Rnd2 and Rnd3 was visualized as described in (**A**). (**C**) Rnd1–Rnd3 were visualized with a rabbit anti-Myc antibody followed by a TRITC-conjugated anti-rabbit antibody. Ezrin was visualized with a mouse anti-ezrin antibody followed by an FITC-conjugated anti-mouse antibody. Filamentous actin was visualized with Alexa Fluor 350-conjugated phalloidin. The bar represents 20 μm.

employed, since these GTPases have been shown to be constitutively GTP-bound in the wild-type state [26,37]. Cells expressing Rnd GTPases lost the majority of their cytoplasmic actin filaments; moreover, many cells looked retracted and attained an arborized or spiky phenotype (see in particular the Rnd2- and Rnd3-transfected cells in Figure 4B). Frequently, the Rnd-expressing cells formed dorsal microvilli, suggesting that the GTPases induced a relocalization of actin from stress fibres to dorsal protrusions. Members of the ERM family of proteins, such as ezrin, have been shown to be localized in microvillar

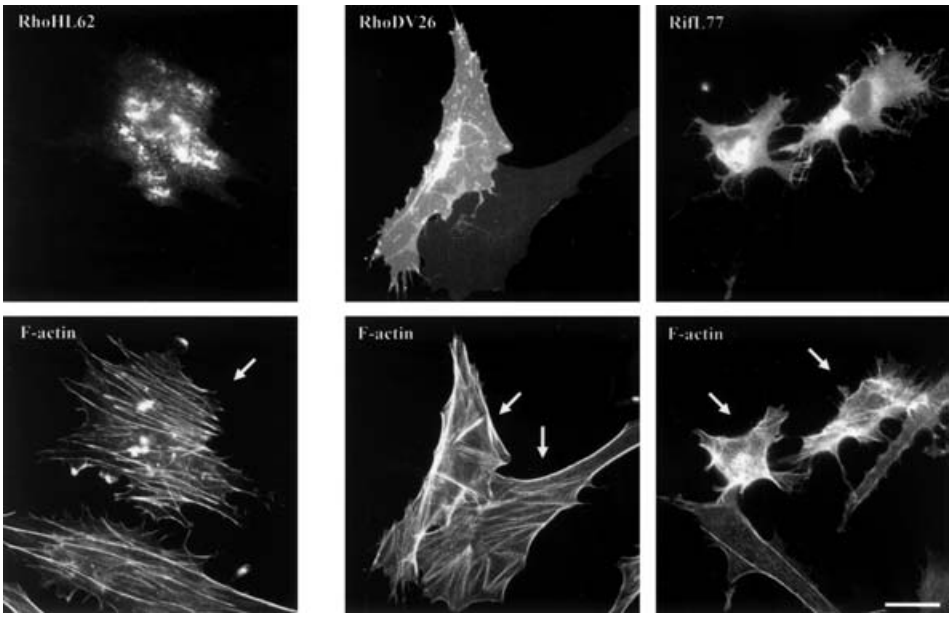


Figure 5 Effects of RhoH and RhoD subfamily members on the organization of the actin filament system in PAE/PDGFRβ cells

The subcellular localization of transiently transfected RhoHL62 and RhoDV26 was visualized by means of an N-terminal enhanced green fluorescent protein tag. RifL77 was visualized with a mouse anti-Myc antibody followed by an FITC-conjugated anti-mouse antibody. Filamentous actin was visualized with TRITC-conjugated phalloidin. Arrows indicate GTPase-expressing cells. The bar represents 20 μm.

protrusions, in particular in epithelial cells, but also in fibroblasts [41,42]. For this reason, cells expressing Rnd GTPases were co-stained for ezrin and, indeed, the Rnd-containing protrusions also contained ezrin (Figure 4C).

RhoH, RhoD and Rif

Transfection of RhoHL62 into PAE/PDGFRβ cells did not have any obvious effects on the organization of the actin filament system (Figure 5). In contrast, transfection with RhoDV26 or RifL77 caused a profound reorganization of the actin filament system (Figure 5). Both GTPases induced very long and flexible filopodia that emerged like octopus tentacles from the cell body. In addition, these GTPases had a dramatic effect on the organization of the actin filament system, a response most pronounced in RifL77-expressing cells, where the cell body was full of actin filament bundles (Figure 5).

RhoBTB and Miro

RhoBTB1 and RhoBTB2 were transfected into PAE/PDGFRβ cells as described above and their effects on the organization of the actin cytoskeleton were examined. These GTPases are thought to function as constitutively GTP-bound proteins since, similar to the Rnd GTPases, they contain amino acid residues in the positions critical for GTP hydrolysis that are likely to impair the GTPase activity of the proteins. Ectopic expression of the RhoBTBs had only a moderate influence on the organization of the actin filament system (Figure 6). Examination of RhoBTB-expressing cells demonstrated that both RhoBTB1 and RhoBTB2 were organized into vesicular structures in the cytoplasm. Co-staining with markers for lysosomes and the Golgi apparatus indicated that the RhoBTBs are not present in these compartments (results not shown). Constitutively active mutants of Miro-1 (Miro-1V13) and Miro-2 (Miro-2V13) induced a collapse of the mitochondrial network (Figure 6), but had no effect on the actin filament system, in agreement with earlier reports [7].

Specificity of GTPase-binding domains

GST fusion proteins of the CRIB domains of WASP, PAK1B and rhotekin have been widely used in pull-down assays to study activation of Cdc42, Rac and Rho GTPases respectively [43]. This assay for GTPase activation relies on the fact that the fusion proteins only recognize the GTP-bound, activated, conformation of the Rho GTPases. In order to see which of the Rho GTPases have the capacity to bind to the respective GST fusion constructs, Cos7 cells were transfected with active (i.e. GTP-bound) versions of the Rho GTPases. The cells were lysed and subjected to a pull-down assay in which the Rho GTPases were precipitated with the GST fusion proteins and their presence in the precipitates was determined by immunoblotting. WASP has been shown to bind to the Cdc42 subfamily [44], and it was found that Cdc42, TCL and TC10 bound to GST–WASP-CRIB (Figure 7A). In addition, Rac1, Rac2 and Rac3 bound to GST–WASP-CRIB in this assay, similar to what has been found with Rac1 (Figure 7A) [45]. Moreover, all of these GTPases, as well as Wrch-1, bound to GST–PAK-CRIB. RhoA, RhoB, RhoC and also TC10 (and to a minor extent TCL, RhoH, RhoD and Rif) bound to GST–rhotekin (Figure 7A).

The results from the pull-down assay can be compared with those from a yeast two-hybrid system experiment in which a number of known Cdc42 effectors were tested for binding to the four additional members of the Cdc42 subfamily. Effectors fused to the GAL4 activation domain were transformed into yeast strains expressing GAL4-DB fusions of TC10L75, TCLL79, ChpL89 or Wrch-1L107 using the procedure described previously [34]. WASP bound only to TCLL79 in this assay, whereas N-WASP (neuronal WASP) bound to TC10L75, TCL79 and ChpL89. The most obvious difference concerned ChpL89, which did not bind any GST fusion protein in the pull-down assay, but bound strongly to N-WASP, MLK3 (mixed-lineage kinase 3), PAR6 (partitioning

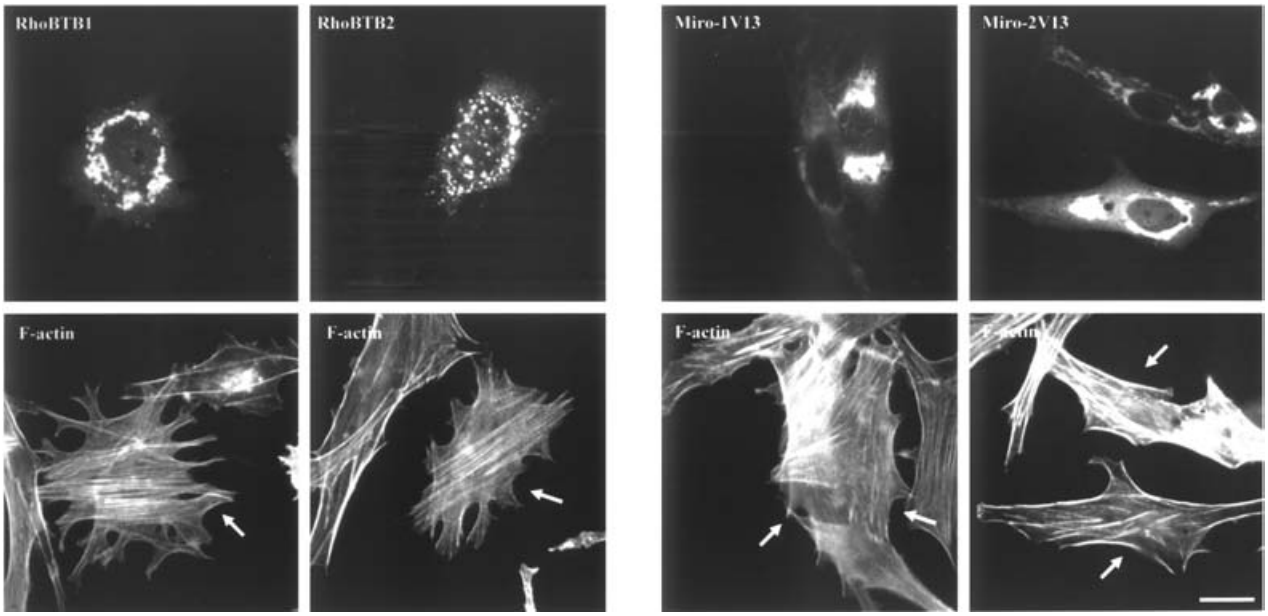
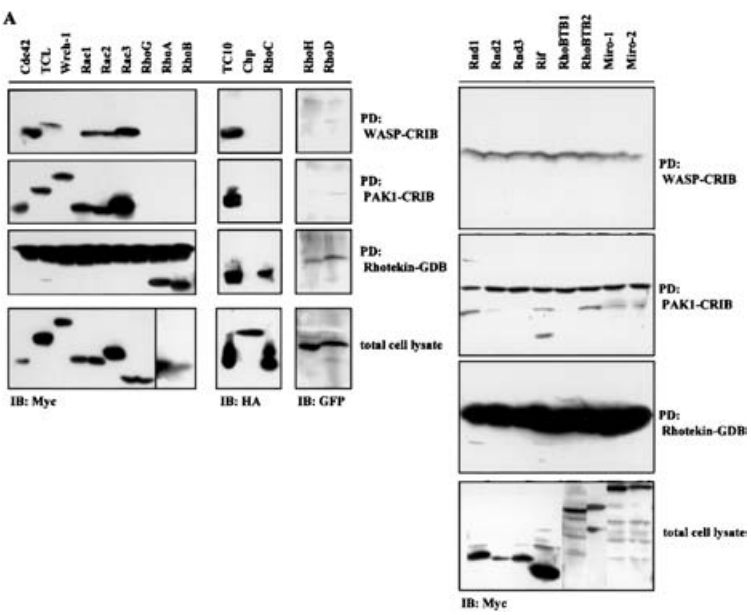


Figure 6 Organization of RhoBTB and Miro subfamily members in PAE/PDGFRβ cells

The subcellular localization of transiently transfected RhoBTB1, RhoBTB2, Miro-1V13 and Miro-2V13 was visualized with a mouse anti-Myc antibody followed by an FITC-conjugated anti-mouse antibody. Filamentous actin was visualized with TRITC-conjugated phalloidin. Arrows indicate GTPase-expressing cells. The bar represents 20 μm.



B

	TC10L75	TCLL79	ChpL89	Wrch-1L107
WASP	-	++	-	-
N-WASP	++	++	++	-
MLK3	-	-	++	-
CIP4	++	++	-	-
PAR6	++	++	++	(+)
Spec1	-	-	-	-
p50RhoGAP	++	++	-	-
PAK1B	++	++	++	++
PAK4	++	++	++	-

Figure 7 Interactions between Rho GTPases and some effector proteins

(**A**) The presence of tagged GTPases in precipitates of GST–WASP-CRIB, GST–PAK-CRIB and GST–rhotekin was detected by immunoblotting (IB) using antibodies specific for the respective epitope tags [Myc, HA or EGFP (enhanced green fluorescent protein)]. PD denotes pull-down with the respective GST fusion protein. (**B**) Results of yeast two-hybrid assays for the interaction of Cdc42 subfamily members with known Cdc42-binding proteins. The interaction of GAL4 activation domain fusion proteins of the Cdc42 effectors with GAL4-DB–TC10L75, GAL4-DB–TCLL79, GAL4-DB–ChpL89 or GAL4-DB–Wrch-1 in the yeast two-hybrid system was assessed. Colonies growing on selective media as described in the Experimental section were assayed for β-galactosidase activity. Maximum activity is scored as ++, whereas (+) denotes barely detectable activity. MLK, mixed-lineage kinase; CIP, Cdc42-interacting protein; PAR, partitioning defective.

defective 6), PAK1B and PAK4 in the yeast two-hybrid assay (Figures 7A and 7B). The reason for this discrepancy is not clear, but the kinetics of the interaction between ChpL89 and potential effector proteins might not allow stable complex formation, which is necessary for effective precipitation with GST fusion proteins.

DISCUSSION

Currently available information from the human genomic sequencing effort suggests the presence of 22 genes for Rho GTPases. In mice, two expressed pseudogenes for RhoA have been identified.

Since they are expressed, they might very well participate in cell signalling [47]. The RhoBTB subfamily actually comprises three members; however, the putative GTP-binding domain of RhoBTB3 differs substantially from all known GTP-binding domains, making it unlikely that it functions as a GTPase domain and, hence, the gene product cannot be categorized as a Rho GTPase [33]. RhoBTB1 and RhoBTB2 most probably have a function distinct from that of the classical Rho GTPases: they have no obvious role in organizing the actin filament system. BTB domains are common in proteins with roles in transcriptional regulation, for instance during embryogenesis. This could imply that RhoBTBs have a role in transcriptional regulation [39]. However, the localization of RhoBTB1/2 in vesicles might suggest another role for these proteins. RhoBTB1/2 might, for instance, participate in endocytosis. Several Rho family members are involved in endocytosis: RhoA and Rac1 have roles in internalization of the transferrin receptor [48], whereas RhoD and RhoB participate in the intracellular trafficking of endosomes [27,28,40].

Cdc42 has been suggested to induce filopodia; however, the most prominent response in cells ectopically expressing Cdc42 is the formation of lamellipodia and very thick bundles of actin filaments. As noted previously, the formation of the lamellipodia occurs via Rac [46]. Cells transfected with the transforming mutant Cdc42L28 induce filopodia rather than lamellipodia (P. Aspenström, unpublished work). This Cdc42 mutant can undergo spontaneous GDP/GTP exchange in the absence of GEFs [49], indicating that the formation of filopodia requires a GTPase with an intact ability to cycle between GDP- and GTP-bound conformations. The other Cdc42 family members did not induce filopodia, with the exception of Wrch-1, which induced a rather extreme spiky phenotype. TCL-expressing cells developed lamellipodia, but these lamellae also had focal adhesion-like assemblies at the cell periphery. TC10 and Chp, in contrast with what has been described previously [23,24], did not induce filopodia, at least not in the PAE cells employed in the present studies. Thus Cdc42, TCL, Rac1–Rac3 and RhoG all triggered the formation of lamellipodia; however, the width and the organization of the lamellae formed differed considerably between the different GTPases, suggesting that different Rho GTPases have both specific and overlapping roles in the formation of lamellipodia.

The formation of long filopodia was a dominant effect in cells constitutively expressing Rif, and to some extent RhoD. Interestingly, Rnd-expressing cells also had filopodia, but in this case on the dorsal side of the cells rather than at the cell periphery. These filopodia also contained ezrin and thus resembled microvilli [42]. These observations demonstrate the existence of several categories of protrusions that are likely to be functionally distinct and to require the activities of distinct members of the Rho GTPase family.

The CRIB domains of WASP and PAK, as well as of rhotekin, have been used to study the activation of Rho GTPases [43]. The observations in the present study suggest that each of these GST fusion proteins has a broader specificity than thought previously. Our data demonstrate that the GST–WASP-CRIB fusion protein is not specific for Cdc42. This notion is of importance, since it demonstrates that the fusion proteins can be used to study activation of several different Rho GTPases. The GST–WASP-CRIB fusion protein has been used for immunohistochemical and immunocytochemical studies in order to stain specifically activated Cdc42 [50]. However, it binds to TCL, TC10, Rac1, Rac2 and Rac3 in addition to Cdc42, something that needs to be taken into consideration in the interpretation of studies employing GST–WASP-CRIB in immunohistochemistry.

According to currently available sequence information, the family of Rho GTPases consists of 22 family members in mammalian cells. The work described in the present study has aimed to compare the specific effects elicited by the Rho GTPases on the organization of the actin filament system. However, this group of proteins also regulates cellular processes such as gene transcription, cell cycle progression, intracellular trafficking and cell survival. It will be a future challenge to establish the contribution of each family member in these cellular processes.

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