RhoGDI-binding-defective mutant of Cdc42Hs targets to membranes and activates filopodia formation but does not cycle with the cytosol of mammalian cells

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We have identified a mutant of the human G-protein Cdc42Hs, R66E, that fails to form a detectable complex with the GDP-dissociation inhibitor RhoGDI in cell-free systems or in intact cells. This point mutant is prenylated, binds guanine nucleotide and interacts with GTPase-activating protein in a manner indistinguishable from wild-type Cdc42Hs. Immunofluorescence localization studies revealed that this RhoGDI-binding-defective mutant is found predominantly in the Golgi apparatus, with a staining pattern similar to that of the wild-type protein. However, unlike wild-type Cdc42Hs, which is distributed in both the microsomal membrane and cytosolic fractions, studies using differential centrifugation show that prenylated R66E Cdc42Hs is found exclusively in association with lipid bilayers. Additionally, whereas the overexpression of RhoGDI results in an apparent translocation of wild-type Cdc42Hs from the Golgi

apparatus into the cytosol, identical RhoGDI-overexpression conditions do not alter the Golgi localization of the R66E mutant. Furthermore, overexpression of this RhoGDI-binding-defective mutant of Cdc42Hs seems to activate redistribution of the actin cytoskeleton and filopodia formation in fibroblasts in a manner indistinguishable from the wild-type protein. Taken together, these results suggest that the interaction of Cdc42Hs with RhoGDI is not essential for proper membrane targeting of nascent prenylated Cdc42Hs in mammalian cells; neither is this interaction an essential part of the mechanism by which Cdc42Hs activates filopodia formation. However, it does seem that redistribution of Cdc42Hs to the cytosolic compartment is absolutely dependent on RhoGDI interaction.

Key words: GTPase, prenylation, targeting.

INTRODUCTION

Like other Ras-related small GTP-binding proteins, the Rho subfamily of GTPases (Rho, Rac, Cdc42 and TC10) function as molecular switches to regulate diverse physiological processes within mammalian cells. Rho-family proteins are involved in the regulation of cell polarity, motility and cell cycle progression and are also necessary for Ras-mediated cellular transformation [1–3]. They are coupled to a variety of proteins that regulate their GTP-binding/GTPase cycle [such as GTPase-activating proteins (GAPs), GDP-dissociation inhibitors (GDIs) and guanine nucleotide exchange factors (GEFs)] and to a variety of effectors that potentially link them to diverse biological processes. The mechanisms by which the specificity of signalling through these various effector molecules is accomplished is probably complex and not yet well understood. Although there is evidence that GEFs are important determinants in the specificity of downstream signalling [4], the mechanisms of upstream regulation that control the specificity of activation of Rho-family signal transduction cascades remain an active area of investigation. It is likely that control of the proper intracellular distribution of this family of GTPases is one of the important factors in determining effector interaction.

Changes in intracellular localization are observed for many of the Ras-related GTPases in response to the cycle of GTP binding and hydrolysis [5–10]. Additionally, many of these GTPases must be localized to lipid bilayers to mediate their associated physiological effects [11,12]. The membrane association of small GTPases is dependent on the covalent attachment of a C₁₅ or C₂₀ isoprenoid group via a thioether linkage to a C-terminal cysteine residue [13]. The Rho family of GTPases is prenylated by the

cytosolic heterodimeric prenyltransferase, geranylgeranyltransferase I (GGTase I) [14,15]. The addition of the C₂₀ isoprenoid group renders the GTPase hydrophobic and capable of stably interacting with lipid bilayers.

Several observations in the literature show changes in the localization of Rho-related GTPases in response to stimuli. For example, lysophosphatidic-acid-induced shape changes in N1E-115 neuronal cells are accompanied by the translocation of RhoA from the cytosol to the cell periphery [16]. If membrane localization of RhoA is prevented by inhibition of prenylation, cytoskeletal contraction is blocked in these cells. Interestingly, lysophosphatidic acid also induces translocation of the inactive N19RhoA mutant from the cytosol to the cell periphery, suggesting that translocation of RhoA in these neuronal cells can occur independently of RhoA activation. Stimulation of membrane ruffling in MDCK (Madin–Darby canine kidney) cells by phorbol ester or hepatocyte growth factor results in a translocation of RhoA to the membrane ruffling area [17]. RhoA also moves from the cytosol to cell–cell adhesion sites when MDCK cells are transferred from a low-Ca²⁺ to a normal Ca²⁺ medium and to cleavage furrows during cytokinesis in Swiss 3T3 cells [17]. In addition, there is an increase in membrane-associated Cdc42Hs when monocytes (such as U937 cells) differentiate into macrophages [18]. These and other studies provide clear evidence that translocation of Rho GTPases from the cytosol to the membrane is closely correlated with changes in cytoskeletal structure [19–21].

Translocation of Rho GTPases from the cytosol to membrane compartments not only is correlated with changes in cell shape but also seems to have a role in other Rho-family-regulated processes. For example, on stimulation of neutrophils, Rac is

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GEF, guanine nucleotide exchange factor; GGTase I, geranylgeranyltransferase I; WT, wild-type.

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released from interaction with RhoGDI in the cytosol and becomes associated with the membrane fraction [22–25]. This translocation to the membrane fraction is also dependent on a heat- and trypsin-labile component, suggesting that membrane bound proteins, possibly GEFs, have a key role in the translocation of Rac to the membrane in response to stimuli [25]. A change in the subcellular distribution of Rho GTPases also occurs during cell cycle progression. RhoA is detected in the membrane fraction of growth stimulated rat thyroid FRTL-5 cells at G_0 , is eliminated from the membrane at mid- G_1 and returns to the membrane during late G_1 [26,27]. Although changes in the subcellular localization of Rho-related GTPases seem to be an important component in a variety of Rho-related physiological responses, little is known about the mechanisms by which such a change in localization results in a physiological effect (such as changes in cell morphology or cell cycle progression); neither is it known how these GTPases move between cellular compartments in a regulated fashion.

The foregoing observations suggest that membrane localization of Rho-related GTPases is an important part of the mechanism by which these GTPases regulate a variety of cellular events. Although the isoprenoid modification of Rho GTPases has a permissive role in membrane association, metabolic labelling studies with [3H]mevalonate have demonstrated that, unlike most other small GTPases, a large proportion of isoprenylated Rho proteins are found in the cytosolic fraction of a variety of cell types [14]. There is a large pool of prenylated Cdc42Hs in the soluble fraction of mammalian cells, suggesting that the geranylgeranyl group has a functional significance in addition to stabilizing the interaction of the protein with the lipid bilayer. In fact, the isoprenoid group is essential in the binding of Rho proteins to their GDP-dissociation inhibitor, RhoGDI [28–30]. Although the interaction of RhoGDI with Rho proteins stabilizes the inactivated GDP-bound form of the GTPase, this accessory protein might have an additional regulatory role by masking the hydrophobic prenyl group from the hydrophilic milieu of the cytosol, thus allowing the GTPase to be retained within the cytosolic compartment of the cell.

Here we describe studies aimed at establishing the role of RhoGDI in regulating the subcellular localization and function of Rho-family GTPases. They exploit a point mutation of Cdc42Hs that is incapable of interacting with RhoGDI in assays *in vitro* and in intact cells, but is normal with regard to its ability to bind guanine nucleotide, to undergo GAP-mediated hydrolysis of GTP and to be prenylated, providing a tool that allows us to examine the importance of RhoGDI interaction on Cdc42Hs localization and function.

MATERIALS AND METHODS

Epitope tagging and mutagenesis

cDNA species encoding Cdc42Hs and RhoGDI were modified by the addition of N-terminal epitope tags [for Cdc42Hs, Myc = EQKLISEEDL; for RhoGDI, FLAG = DYKDDDDK (single-letter amino acid codes)] by PCR with Deep Vent Polymerase (New England Biolabs) and pCMV5Cdc42Hs and pCMV5RhoGDI as templates. Myc-tagged Cdc42Hs was used as the template to generate all point mutations. Mutations were introduced into the Cdc42Hs cDNA by means of overlap extension PCR [31]. The DNA sequence of all constructs was verified by DNA sequencing with ThermoSequenase (Amersham) to verify that no spurious PCR-induced mutations had been introduced into the coding sequences of mycCdc42Hs or FLAGRhoGDI. Constructs were subcloned into pET17b for

bacterial expression and into pCMV5 and pRK5 for expression in mammalian cells.

Expression of recombinant MycCdc42Hs, FLAGRhoGDI and His₆Cdc42HsGAP in *Escherichia coli*

Expression of MycCdc42Hs and FLAGRhoGDI was induced in *E. coli* BL21(DE3)pLysS as described previously [32]. A cDNA construct of His₆GAP in pET15b was generously provided by Dr Richard Cerione (Cornell University, Ithaca, NY, U.S.A.). Bacterial lysates expressing His₆GAP were prepared by the method described previously [32] with the exception that bacteria were lysed in His₆GAP lysis buffer [20 mM Tris/HCl (pH 7.5)/2.5 mM MgCl₂/0.1 mM dithiothreitol/100 mM NaCl]. Wild-type (WT) and mutant MycCdc42Hs forms were quantified in bacterial lysates by immunoblot analysis with an affinity-purified goat anti-rabbit IgG (11.9 μ Ci/ml, ICN) and anti-Cdc42Hs (Santa Cruz) as primary antibody as described previously [14,33]. Comparison of immunoreactive Cdc42Hs in crude bacterial lysates with purified His-tagged Cdc42Hs (Cytoskeleton) permitted determination of the concentration of MycCdc42Hs expressed in the bacterial lysates. The concentration of FLAGRhoGDI in crude bacterial lysates was similarly determined by comparing ^{125}I -IgG immunoblot signals from affinity-purified and quantified preparations of FLAGRhoGDI with the bacterial lysates. Bound ^{125}I -IgG was detected by exposing the blots to Kodak X-Omat AR film; radioactivity was quantified by scanning the autoradiographs with a Microtek Scanmaker III scanner and analysing the scanned images with NIH Image 1.61 software.

[^{32}P]GTP-binding overlay assay

Binding of guanine nucleotides to WT MycCdc42Hs and to all mutants was determined by blot overlay assay as described previously [34]. Approximately equal amounts (3.75 μ g) of each recombinant MycCdc42Hs were used in this assay.

Preparation of crude cytosol from rat brain

One adult rat brain was extracted and homogenized in 2 ml of ice-cold homogenization buffer [50 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/0.2 mM PMSF/0.1 mM leupeptin]. The cytosol was separated from the membranes by centrifugation at 75000 *g* for 30 min and stored at $-80^{\circ}C$ until use.

Prenylation of recombinant MycCdc42Hs

Equal amounts (0.25 μ g) of MycCdc42Hs WT and mutant recombinant proteins were incubated with a crude source of GGTase I (rat brain cytosol) in the presence of 50 mM Tris/HCl (pH 7.7)/20 mM KCl/5 mM MgCl₂/25 μ M ZnCl₂/1 mM dithiothreitol/0.5 mM Zwittergent 3–14 as described by Zhang et al. [35] in a final reaction volume of 50 μ l. Prenylation reactions were initiated by the addition of the prenyl substrate, [3H]geranylgeranyl pyrophosphate (15 Ci/mmol; American Radiolabeled Chemical Corp.) and left to proceed for 1 h at $37^{\circ}C$. Reactions were terminated after 1 h by the addition of SDS sample buffer and were separated by SDS/PAGE [12.5% (w/v) gel] [36]. The gel was Amplified (Amersham), dried and subjected to fluorography.

Cell-free assay for the formation of Cdc42Hs–RhoGDI complexes

Aliquots of *E. coli* lysate containing equal amounts of Myc-tagged WT and mutant Cdc42Hs proteins (0.5 μ g) were prenylated as described above. After a 1 h incubation at $37^{\circ}C$,

SDS sample buffer was added to half of the reaction and the extent of MycCdc42Hs prenylation was assessed by SDS/PAGE and subsequent fluorography of the dried gel. Recombinant FLAGRhoGDI (7 μ g) was added to the remaining half of the prenylation reaction in the presence of 4 mM NaPO₄ (pH 7.5)/0.4 mM MgCl₂/80 μ M GDP/60 mM NaCl in a final reaction volume of 100 μ l. After a 1 h incubation at 37 °C, the reactions were added to 100 μ l of a 1:1 slurry of anti-FLAG M2 affinity gel (Sigma) which had been prewashed twice with 0.1 M glycine, pH 3, and four times with phosphate wash buffer (10 mM NaPO₄/1 mM MgCl₂/200 μ M GDP/0.15 M NaCl). Samples were mixed end-over-end for 30 min at 4 °C and the resin was collected by centrifugation at 8160 g for 1 min. The supernatant solution was removed and the resin was washed three times with phosphate wash buffer. FLAGRhoGDI complexes were then eluted by addition of 0.1 M glycine, pH 3, to the resin. Eluates were immediately neutralized by the addition of 1 M Tris/HCl, pH 8. The eluates were then mixed with SDS sample buffer and subjected to SDS/PAGE [12.5% (w/v) gel]. MycCdc42Hs that was co-eluted with RhoGDI was detected as [³H]geranylgeranyl-labelled protein on the fluorogram of the Amplified and dried gel.

GAP-mediated hydrolysis of GTP

Bacterial lysates expressing recombinant MycCdc42Hs (approx. 3.7 μ g) were incubated with [γ -³²P]GTP (22.5 μ Ci at 4500 Ci/mmol; ICN) in the presence of 30 mM Hepes (pH 8)/0.6 mM dithiothreitol/3 mM MgCl₂/60 μ M GDP/10 mM EDTA (pH 8)/10 mM ATP/0.1 mM adenosine 5'-[β , γ -imido]triphosphate/100 mM NaCl (final reaction volume 75 μ l) for 20 min at 23 °C. High concentrations of MgCl₂ and unlabelled GTP (10 mM each) were then added to the loading reactions to terminate loading of the radioactive nucleotide and to decrease the dissociation of nucleotide that was already bound. Triplicate aliquots equal to 5% of the total reaction volume were removed and diluted in 10 ml of ice-cold dilution buffer [20 mM Tris/HCl (pH 7.5)/100 mM NaCl/10 mM MgCl₂] and rapidly filtered through BA85 (Schleicher and Schuell) nitrocellulose filter discs on a 1225 Millipore sampling manifold. Each filter was then washed with 40 ml of ice-cold dilution buffer to decrease non-specific binding of [³²P]GTP to the filter disc. These samples represent the binding of [³²P]GTP at zero time. Aliquots representing 23% of the volume of the initial loading reaction were then added to a hydrolysis reaction mix that consisted of 7 μ l of a crude bacterial lysate expressing His₆-tagged Cdc42HsGAP (plus GAP condition) or 7 μ l of GAP lysis buffer (minus GAP condition) in the presence of 20 mM Tris/HCl (pH 7.5)/20 mM MgCl₂/10 mM GTP/100 mM NaCl/1 mM adenosine 5'-[β , γ -imido]triphosphate/1 mg/ml BSA. Aliquots were removed from the replicate hydrolysis reactions after 1, 2, 3 and 10 min and were processed as described above for the zero-time points. Aliquot volumes were calculated so that equivalent amounts of Myc Cdc42Hs were sampled for each time point (i.e. 0 compared with 1, 2, 3 and 10 min). Radioactivity bound to the filters was quantified by mixing the dried filters with scintillation fluor and analysing them in a liquid-scintillation counter.

Subcellular distribution of MycCdc42Hs expressed in mammalian cells

HEK-293 cells were plated at a density of 7.5×10^5 per 60 mm dish in growth medium [α -minimal essential medium/10% (v/v) heat-inactivated fetal bovine serum (FBS)] approx. 20 h before transfection. All transfections were performed with the calcium phosphate precipitation technique [37]. Cultures were trans-

fected with DNA mixtures containing 35 μ g of pCMV5, 35 μ g of pCMV5:MycCdc42Hs(WT) alone, 35 μ g of pCMV5:MycCdc42Hs(R66E) alone, 30 μ g of pCMV5:FLAGRhoGDI alone or the MycCdc42Hs constructs individually in combination with pCMV5:FLAGRhoGDI. At 3 h after the addition of DNA, cultures were glycerol-shocked by the addition of 15% (v/v) glycerol in PBS for 30 s. Cultures were then left to recover for 20–24 h under normal growth conditions [complete medium, 37 °C, air/CO₂ (19:1)]. Cultures were then harvested in PBS and homogenized in 0.3 ml of lysis buffer [100 mM Tris/HCl (pH 7.4)/0.1 mM GDP/1 mM MgCl₂/50 mM NaF/1 mM PMSF]. Nuclei and unbroken cells were separated from the rest of the homogenate by centrifugation at 500 g for 5 min at 4 °C. Cytosol and microsomal membranes were then separated by high-speed centrifugation of the low-speed supernatant fraction (100 000 g for 30 min at 4 °C). Cytosolic and membrane fractions were analysed by SDS/PAGE and Western blotting of the gels transferred electrophoretically to PVDF with anti-Myc antibody and goat anti-mouse ¹²⁵I-IgG to detect MycCdc42Hs. Autoradiographs were quantified with a Microtek Scanmaker III scanner and NIH Image 1.61 software.

Co-precipitation assay for the interaction of co-expressed MycCdc42Hs and FLAGRhoGDI in intact mammalian cells

Cytosol was prepared as described above from HEK-293 cell cultures expressing WT MycCdc42Hs and R66E MycCdc42Hs alone or in combination with FLAGRhoGDI. Half of the cytosol (150 μ l) obtained from three 60 mm dishes combined was mixed with 100 μ l of a 1:1 slurry of anti-FLAG M2 affinity resin prepared as described above and rotated end-over-end for 30 min at 4 °C. The resin was washed and FLAGRhoGDI-MycCdc42Hs complexes were eluted as described for the co-precipitation assay *in vitro*. Cytosol (a measure of total MycCdc42Hs and FLAGRhoGDI loaded on the anti-FLAG resin) and low-pH glycine eluates were separated by SDS/PAGE [12.5% (w/v) gel] and were subsequently transferred by electroblotting to PVDF membranes and blotted with anti-Myc antibody and goat anti-mouse ¹²⁵I-IgG or anti-RhoGDI antibody and goat anti-rabbit ¹²⁵I-IgG. Immunoreactive proteins were then detected by autoradiography of the Western blots.

Prenylation band-shift assay

HEK-293 cells were grown and transfected as described above with 30 μ g of pCMV5:MycCdc42Hs(WT), 30 μ g of pCMV5:MycCdc42Hs(WT), 30 μ g of pCMV5:MycCdc42Hs(R66E) or 30 μ g of pCMV5:MycCdc42Hs(C188S). Immediately after the glycerol shock, 10 μ M lovastatin was added to the medium of selected dishes. Approximately 20 h after transfection, cells were harvested and cytosolic and microsomal membrane fractions were separated as described above. Samples were separated by SDS/PAGE [12.5% (w/v) gel] and were subsequently transferred to PVDF membranes and Western blotted with anti-Myc antibody and ¹²⁵I-labelled goat anti-mouse IgG.

Immunofluorescence of HEK-293 cells

HEK-293 cells were transfected as described above with 30 μ g of pCMV5:MycCdc42Hs(WT) or 30 μ g of pCMV5. Approximately 18 h after transfection, cells were detached with trypsin/EDTA and washed once in growth medium [α -minimal essential medium/10% (v/v) heat-inactivated FBS] and twice with DMEM (Dulbecco's modified Eagle's medium) (Gibco/BRL). Cells were finally resuspended in DMEM with 5% (v/v) FBS. Approximately 200 000 cells were plated into 60 mm dishes

containing 1.8 cm (diameter) glass coverslips that had been coated with 10 $\mu\text{g}/\text{ml}$ fibronectin (Gibco/BRL) for 16 h at 4 °C. Cells were incubated at 37 °C in air/CO₂ (19:1) for 1 h to allow attachment to the fibronectin-treated coverslips. After re-attachment, cells were fixed in 3.7% (w/v) paraformaldehyde and permeabilized in 0.05% (v/v) Triton X-100. Cells were then blocked in PBS with 5% (w/v) dried milk and overexpressed Myc-tagged Cdc42Hs was detected by staining with anti-Myc antibody and tetramethylrhodamine β -isothiocyanate-labelled goat anti-mouse IgG. The Golgi apparatus was then stained with FITC-labelled lectin from *Lens culinaris* (Sigma) as described previously [38]. Coverslips were mounted on glass slides with fluorescent mounting media (Dako). Slides were viewed and photographed on a Zeiss Axiovert S100 fluorescence microscope at 60 $\times$ magnification.

Immunofluorescence of NIH 3T3 cells

NIH 3T3 cells were plated at a density of 1.5×10^5 cells/100 mm dish in growth medium [DMEM containing 10% (v/v) FBS]. Three glass coverslips (1.8 cm coated with 20 $\mu\text{g}/\text{ml}$ laminin) were added to each 100 mm dish at the time of plating. Approximately 20 h later the cultures were transfected with Eugene 6 transfection reagent (Roche). Cultures were transfected with DNA/Eugene mixtures containing 2.0 μg of pRK5/12 μl Eugene; 2.0 μg of pRK5:MycCdc42Hs(WT) plus 12 μl of Eugene; 2.0 μg of pRK5:MycCdc42Hs(R66E) plus 12 μl of Eugene; 2.0 μg of pRK5:MycCdc42Hs(WT) plus 2.0 μg of pRK5:FLAGRhoGDI and 24 μl of Eugene; or 2.0 μg of pRK5:MycCdc42Hs(R66E) plus 2.0 μg of pRK5:FLAGRhoGDI and 24 μl Eugene as indicated. In brief, DNA/Eugene mixtures were incubated at room temperature (22 °C) for 15 min, then added to cultures. Cells were left to grow for 24 h at 37 °C under air/CO₂ (19:1) in DMEM with 10% (v/v) FBS and were then serum-starved for 24 h in DMEM/0.5% (v/v) FBS. Coverslips were removed from the 100 mm culture dishes and were fixed and permeabilized for 10 min at 37 °C in freshly prepared paraformaldehyde/Triton X-100 solution [4% (w/v) paraformaldehyde/0.2% (v/v) Triton X-100/60 mM Pipes/25 mM Hepes/10 mM EGTA/3 mM MgCl₂ (pH 6.1)]. Actin was then stained with 100 nM Rhodamine-Phalloidin (Cytoskeleton). Cells were blocked in PBS with 3% (w/v) BSA; overexpressed Myc-tagged Cdc42Hs was detected by staining with anti-Myc antibody (9E10 Santa Cruz Biotechnology) and FITC-labelled goat anti-mouse IgG (Sigma). Coverslips were mounted with Prolong anti-fade mounting medium (Molecular Probes) and were viewed with a 100 $\times$ objective on a Zeiss Axioplan 2 fluorescence microscope.

RESULTS

Characterization of guanine nucleotide binding and prenylation of MycCdc42Hs point mutations

In an attempt to explore the significance of Cdc42Hs interaction with RhoGDI in the initial membrane targeting and localization of Cdc42Hs, we sought to identify a mutant of Cdc42Hs that was incapable of interacting with RhoGDI but was normal in its ability to bind guanine nucleotide and to be prenylated by GGTase I. Point mutations were introduced into the surface-exposed loop regions of Cdc42Hs. Binding of guanine nucleotide to the mutant proteins was assessed by a blot overlay assay of recombinant proteins (Figure 1). As shown in Figure 1, all of the mutants designed could be synthesized and isolated for further evaluation. As shown by the [³²P]GTP binding (gel overlay

assay), the T17N and D118H mutants are guanine-nucleotide-binding mutants and are defective in their ability to bind nucleotide [1,39]. All of the other mutants bind guanine nucleotide at least as well as WT MycCdc42Hs. Interestingly, T35A and Y40K Cdc42Hs seemed to bind [³²P]GTP to an even greater extent than WT Cdc42Hs, even though equal amounts of protein were evaluated in these studies (Figure 1C).

Current understanding suggests that for a small GTPase to localize to a lipid bilayer, the protein must be prenylated [13]. Because it was our goal to understand what role, if any, RhoGDI has in the initial membrane targeting of nascent prenylated Cdc42Hs, it was essential to establish that any mutant used to study the Cdc42Hs–RhoGDI interaction could be prenylated in a manner indistinguishable from that of WT Cdc42Hs. As shown in Figure 1(B), prenylation of each point mutation evaluated in these studies did occur, although there seemed to be a modest decrease in prenylation of the guanine-nucleotide-binding mutants T17N and D118H.

Mutation within the loop 4 domain of Cdc42Hs disrupts the interaction of Cdc42Hs with RhoGDI in a cell-free assay system

To assess the ability of the mutant Cdc42Hs structures summarized in Figure 1 to interact with RhoGDI, we developed a Cdc42Hs–RhoGDI binding assay *in vitro*. *E. coli* lysates expressing Cdc42Hs WT or mutant proteins were prenylated and then incubated with recombinant N-terminal FLAG-epitope-tagged RhoGDI to allow the formation of the Cdc42Hs–RhoGDI complex. The MycCdc42Hs–FLAGRhoGDI complexes were isolated with an anti-FLAG resin. As shown in Figure 2, WT Cdc42Hs interacted with RhoGDI and was isolated with anti-FLAG antibody by virtue of its association with FLAGRhoGDI. As expected, neither T17N nor D118H Cdc42Hs interacted with RhoGDI because they did not bind guanine nucleotide and were therefore incapable of interacting with RhoGDI. In addition, MycRab1B, known to be incapable of interacting with RhoGDI, also failed to associate with FLAGRhoGDI in this assay, confirming that this *in vitro* assay that we developed was a valid assessment of MycCdc42Hs–RhoGDI interaction. The exciting finding in these studies was that one of the loop 4 mutants, R66E Cdc42Hs, was not capable of detectable interaction with FLAGRhoGDI, despite its ability to bind GTP (Figure 1A) or of prenylation by GGTase I [Figure 1B, and internal control for protein loading in binding assay *in vitro* (Figure 2)]. Thus we identified at least one mutant structure of Cdc42Hs that could not interact with RhoGDI and might serve as a tool to assess the importance of this interaction in the localization and function of Cdc42Hs. This finding is consistent with the recent findings of Cerione and colleagues [29], who have solved the crystal structure of the Cdc42Hs–RhoGDI complex at 2.6 Å resolution and have determined that the N-terminal regulatory arm of RhoGDI interacts with the switch II region of Cdc42Hs.

R66E MycCdc42Hs interacts normally with Cdc42HsGAP

To assess whether R66E Cdc42Hs was capable of interacting with other accessory proteins such as GAP, recombinant His₆GAP (a GTPase-activating protein that activates the GTPase activity of Cdc42Hs) was added to [γ -³²P]GTP-loaded MycCdc42Hs. GTP hydrolysis was measured as a function of loss of radioactivity associated with Cdc42Hs owing to release of the γ -labelled ³²P on hydrolysis. As shown in Figure 3, GAP-stimulated hydrolysis of GTP occurred indistinguishably for WT and R66E Cdc42Hs, providing evidence that R66E was fully capable of interacting with GAP.

Figure 1 Cell-free guanine nucleotide binding and prenylation assays

Recombinant WT MycCdc42Hs and mutant proteins were assayed for prenylation and guanine nucleotide binding as described in the Materials and methods section. The top panel is a schematic representation of the location of the point mutations within the three-dimensional structure of Cdc42Hs (Brookhaven Protein Databank accession number 1AJE). (A) Autoradiograph of the [³²P]GTP-labelled MycCdc42Hs in the guanine nucleotide binding overlay assay. The exposure time was 7 h, except for the 'loop 2' panel, which was exposed for 30 min. (B) Fluorograph of the [³H]geranylgeranol ([³H]GG)-labelled MycCdc42Hs. The exposure time was 5 days. (C) Autoradiograph of the [¹²⁵I]-IgG-labelled Western blot, which demonstrates that approximately equal amounts of recombinant MycCdc42Hs were used in each assay condition and also provides a means by which to normalize the [³²P]GTP binding and [³H]geranylgeranol labelling reactions. The exposure time was 7 h. The ratio of [³H]geranylgeranol to [¹²⁵I]-IgG for each mutant normalized to the ratio for the WT protein are as follows: WT, 1.0; Y32K, 1.1; T35A, 1.5; Y40K, 1.43; D63N, 0.92; R66E, 0.85; Q74D, 1.2; E127N, 1.3; K131, 1.4; T17N, 0.54; D118H, 0.81. Results are representative of at least three separate experiments. The positions of molecular mass markers are indicated (in kDa) at the right.

Figure 2 R66E MycCdc42Hs does not co-precipitate with FLAGRhoGDI *in vitro*

Equal amounts of recombinant WT MycCdc42Hs and mutant proteins were geranylgeranylated and co-precipitated with FLAGRhoGDI as described in the Materials and methods section. Upper panel: fluorograph of prenylated MycCdc42Hs, co-precipitated as a complex with FLAGRhoGDI. Lower panel: fluorograph of an aliquot (50%) of the prenylation before the addition of recombinant FLAGRhoGDI. This is therefore representative of the amount of [³H]geranylgeranol ([³H]GG)-labelled MycCdc42Hs that was incubated with FLAGRhoGDI. The exposure times of the upper and lower panels were 6 days and 1 day respectively. The ratios of [³H]geranylgeranol in the glycine eluate to [³H]geranylgeranol in the prenylation reaction normalized to the ratio for the WT MycCdc42Hs are as follows: WT (no GDI), 0; WT, 1.0; Y32K, 0.78; T35A, 0.51; Y40K, 1.4; D63N, 0.81; R66E, 0; Q74D, 2.1; E127N, 1.5; K131Y, 1.1; T17N, 0; D118H, 0; Rab1B, 0. Results are representative of two separate experiments. The positions of molecular mass markers are indicated (in kDa) at the right.

Figure 3 GAP stimulates the hydrolysis of GTP bound to MycCdc42Hs

Equal amounts of recombinant WT and R66E MycCdc42Hs were analysed in a GAP assay as described in the Materials and methods section. After incubation of $[^{32}\text{P}]\text{GTP}$ -labelled MycCdc42Hs with recombinant GAP, reactions were quenched by dilution and filtration on nitrocellulose filters that were dried and quantified in a liquid-scintillation counter. Symbols: □, WT MycCdc42Hs in the absence of GAP; ■, WT MycCdc42Hs in the presence of GAP; ○, R66E MycCdc42Hs in the absence of GAP; ●, R66E MycCdc42Hs in the presence of GAP. Results are means $\pm$ S.D. for triplicate determinations. Where error bars are not visible, they are smaller than the size of the symbol.

Figure 4 R66E MycCdc42Hs does not form a detectable complex with FLAGRhoGDI in intact HEK-293 cells

WT or R66E MycCdc42Hs was transiently co-expressed with or without FLAGRhoGDI in HEK-293 cells as indicated below each panel and co-precipitated with anti-FLAG affinity resin (see the Materials and methods section). (A, B) Autoradiographs from immunoblot analyses performed on aliquots of the cell cytosol before the addition of the anti-FLAG resin. Overexpressed as well as endogenous RhoGDI was detected (A) with a polyclonal antibody against RhoGDI; MycCdc42Hs was detected (B) with monoclonal anti-Myc antibody as primary. (C) Western blot analysis of the glycine eluates of the anti-FLAG resin. MycCdc42Hs that co-precipitated with FLAGRhoGDI and were eluted from the anti-FLAG resin was revealed by detection with anti-Myc antibody. For all blots shown, the detection of bound primary IgGs was accomplished with secondary ^{125}I -labelled IgG. The exposure time of all panels was 64 h. Results are representative of two independent experiments.

R66E MycCdc42Hs fails to form a detectable complex with FLAGRhoGDI in the cytosol of intact cells

Because the goal of identifying a mutant of Cdc42Hs that selectively disrupted the interaction of Cdc42Hs with RhoGDI was to determine whether or not RhoGDI had a role in chaperoning or targeting nascent prenylated Cdc42Hs from the cytosolic prenyltransferase complex to a lipid bilayer, it was important to learn whether or not the R66E mutation in Cdc42Hs disrupted the ability of this protein to interact with RhoGDI in the context of an intact cell (Figure 4). As shown in Figure 4, co-expression of R66E Cdc42Hs with FLAGRhoGDI in HEK-293

Figure 5 Subcellular distribution of MycCdc42Hs in HEK-293 cells

Microsomal membrane and cytosolic fractions were prepared as described in the Materials and methods section from independent cultures expressing WT or R66E MycCdc42Hs in the presence or absence of co-expressed FLAGRhoGDI as indicated. Aliquots of each fraction were subjected to SDS/PAGE and were immunoblotted with anti-Myc antibody as primary and goat anti-mouse ^{125}I -labelled IgG as secondary antibody. The autoradiograph was quantified as described and the percentage of immunodetectable MycCdc42Hs present in each fraction is shown as a function of total cellular MycCdc42Hs. Filled areas represent the amounts of MycCdc42Hs expressed in the microsomal membrane fraction; open areas depict the percentage of the total MycCdc42Hs that was localized to the cytosolic fraction. Results are representative of two independent experiments.

cells did not lead to co-immunoisolation of mutant Cdc42Hs with RhoGDI, in contrast with observations for WT Cdc42Hs. Although the amount of R66E MycCdc42Hs present in the cytosolic fraction of this reaction condition was substantially lower than that of the WT protein co-expressed with FLAGRhoGDI within the same experiment, a 5-fold longer exposure time of the anti-FLAG resin eluates revealed no detectable MycCdc42Hs signal (results not shown). Thus we interpret these findings to indicate that R66E Cdc42Hs did not interact with RhoGDI *in vitro* or *in situ*.

R66E MycCdc42Hs localizes to microsomal membranes

It was of concern to us that the amount of R66E MycCdc42Hs was significantly decreased in the cytosolic fraction (Figure 4) because this might have been due to inefficient transcription, translation or subsequent protein stability in this heterologous cell system. Consequently we examined the subcellular distribution of the R66E mutant of MycCdc42Hs in HEK-293 cells. WT MycCdc42Hs and mutant proteins were expressed alone or in combination with FLAGRhoGDI. When the WT and R66E mutants were expressed alone, they distributed between the cytosolic and membrane fractions in a manner similar to that previously observed for endogenously expressed Cdc42Hs in a variety of mammalian cell types [14]. When FLAGRhoGDI was co-expressed with WT MycCdc42Hs, little or no detectable MycCdc42Hs localized to the microsomal membrane fraction. The opposite phenomenon was observed for R66E MycCdc42Hs. On co-expression with FLAGRhoGDI, the R66E mutant accumulated within the membrane pellet and was only minimally observed within the cytosolic fraction, as noted in Figure 4.

Band-shift assay to assess localization of prenylated MycCdc42Hs

Because the R66E mutant of Cdc42Hs was distributed almost equally between the cytosolic and microsomal membrane fractions when expressed in the absence of FLAGRhoGDI (Figure 5), we wondered whether any of the cytosolic R66E

Figure 6 Band-shift assay to determine the localization of prenylated MycCdc42Hs

WT, R66E or C188S MycCdc42Hs was expressed in HEK-293 cells in the presence or absence of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor lovastatin. Microsomal membranes and cytosol were fractionated as described in the Materials and methods section. Lanes M, membrane fractions; lanes C, cytosolic fractions. Shown is an autoradiograph of a Western blot detecting MycCdc42Hs with anti-Myc antibody as primary and goat anti-mouse 125 I-labelled IgG as secondary. The exposure time was 66 h. Results are representative of two independent experiments. The positions of molecular mass markers are indicated at the right.

Figure 7 Immunofluorescence localization of MycCdc42Hs in HEK-293 cells

WT or R66E MycCdc42Hs was transiently expressed in HEK-293 cells and detected with anti-Myc antibody and tetramethylrhodamine β -isothiocyanate-labelled goat anti-mouse IgG as described in the Materials and methods section. Cells were also labelled with FITC-labelled lectin from *Lens culinaris* to identify the Golgi apparatus. Magnification, $\times 54$.

MycCdc42Hs was prenylated and, if so, how cytosolic prenylated R66E MycCdc42Hs might be stabilized in the hydrophilic cytosol in the absence of interaction with RhoGDI. To determine the prenylation state of WT and R66E MycCdc42Hs in the context of a cell, we employed a band-shift assay in combination with differential centrifugation. As shown in Figure 6, the prenylated WT MycCdc42Hs migrated more rapidly in SDS/PAGE than a prenylation-defective mutant, C188S MycCdc42Hs, providing 'migration markers' to assess the probable prenylation state of R66E MycCdc42Hs. For each form (WT, R66E and C188S), the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor lovastatin was included as a control to verify the migration of the non-prenylated protein. As shown in Figure 6, most of the hetero-

logously expressed WT MycCdc42Hs in the cytosolic fraction was prenylated, although a substantial amount of the protein in this fraction was not modified, on the basis of the co-migration of WT MycCdc42Hs in the cytosol (C) with both the prenylated and non-prenylated forms of the protein. As expected, all of the WT MycCdc42Hs in the membrane fraction (M) was prenylated. In contrast with our findings for WT MycCdc42Hs, all of the R66E MycCdc42Hs found in the cytosolic fraction was non-prenylated (Figure 6). Prenylated R66E MycCdc42Hs was found exclusively in the membrane fraction. These findings suggest that interactions of Cdc42Hs with RhoGDI, which are lost in the R66E mutant, were essential for the stability of prenylated Cdc42Hs in the cytosolic fraction.

Figure 8 R66E MycCdc42Hs stimulates redistribution of actin and filopodia formation in NIH 3T3 cells

WT or R66E MycCdc42Hs was transiently expressed in NIH 3T3 cells in the presence or absence of overexpressed FLAGRhoGDI as indicated. Upper panels: Myc staining of the overexpressed MycCdc42Hs. Lower panels: the same field of cells stained with Rhodamine–Phalloidin to identify actin. The images shown are representative of cell morphologies in three independent experiments.

R66E MycCdc42Hs localizes to the Golgi apparatus

We used an immunofluorescence approach to ascertain the microsomal membrane compartment in which the MycCdc42Hs existed after heterologous expression in HEK-293 cells. Figure 7 shows that the GDI-binding defective mutant of Cdc42Hs (R66E) co-localized with the Golgi marker, FITC-labelled lectin from *Lens culinaris*, in a manner indistinguishable from that of WT MycCdc42Hs.

R66E MycCdc42Hs stimulates the reorganization of actin filaments and filopodia formation in NIH 3T3 cells

The R66E mutant of Cdc42Hs activated filopodia formation and the redistribution of actin filaments in a manner indistinguishable from that of the WT protein when overexpressed in NIH 3T3 cells (Figure 8). Whereas the co-expression of RhoGDI resulted in an apparent redistribution of the overexpressed WT MycCdc42Hs from the Golgi into the cytosol and a marked diminution of activation of filopodia formation and actin redistribution, the localization and functional effects of the R66E mutant were unaffected by co-overexpression of RhoGDI. Expression of FLAGRhoGDI in these cultures was confirmed by Western blot analysis (results not shown).

DISCUSSION

Characterization of a RhoGDI-binding-defective mutant of Cdc42Hs

We have identified the R66E mutant of Cdc42Hs as a RhoGDI-binding-defective mutant. This mutant does not form a stable association with RhoGDI in cell-free (Figure 2) or intact-cell (Figure 4) co-precipitation assays. However, it does bind guanine nucleotide (Figure 1), undergo prenylation by GGase I (Figure 1) and interact with Cdc42HsGAP in a manner that is virtually indistinguishable from that of the WT protein. All of these

retained functions suggest that the mutation of Arg⁶⁶ to Glu⁶⁶ does not grossly affect the overall structural integrity of the GTPase. It therefore seems that this point mutation quite selectively disrupts the interaction between Cdc42Hs and RhoGDI and serves to confirm loop 4 of the GTPase as a region that is crucial in the protein–protein interaction between Cdc42Hs and RhoGDI. These results are consistent with crystallization studies of the Rho–RhoGDI complex and the Cdc42Hs–RhoGDI complex, which predict that the N-terminus of RhoGDI is bound to the switch I and switch II regions of the GTPase [29,40]. Our mutagenesis strategy has therefore provided not only a tool with which to study the functional significance of Cdc42Hs–RhoGDI interaction in terms of targeting Cdc42Hs to an acceptor membrane but has also provided direct confirmation that the switch II region of Cdc42Hs is absolutely required for the stable interaction of Cdc42Hs with RhoGDI.

Characterization of subcellular distribution of R66E MycCdc42Hs

The ability of R66E MycCdc42Hs to localize to the microsomal membrane fraction of HEK-293 cells (Figure 5), and more specifically the Golgi apparatus (Figure 7), demonstrates that the Cdc42Hs–RhoGDI complex is not an essential intermediate between the cytosolic prenyltransferase machinery and the acceptor membrane. Interestingly, the overexpression of RhoGDI in this experiment alters the localization of the prenylated WT MycCdc42Hs. There is an increase in the ratio of cytosolic to membrane WT MycCdc42Hs in the presence of excess RhoGDI. There are at least two possibilities that could explain these results. The excess RhoGDI might serve to solubilize most, if not all, of the membrane-associated Cdc42Hs or it might prevent Cdc42Hs from ever reaching the membrane. The R66E MycCdc42Hs subcellular distribution results are consistent with these two possibilities because this RhoGDI-binding-defective mutant predominantly localizes within the membrane fraction on overexpression of RhoGDI. These results probably reflect an

inability of RhoGDI to solubilize the mutant protein from the membrane or an inability to retain the protein in the cytosol and prevent membrane association.

The question of how a prenylated, hydrophobic GTPase moves from its prenyltransferase complex within the hydrophilic milieu of the cytosol to a lipid bilayer is a question that remains unresolved for most Ras-related GTP-binding proteins. Because a hydrophobic pocket that could accommodate a C₁₅ or C₂₀ isoprenoid chain does not seem to exist within the structure of the Ras-related GTPases [41], it is likely that a second protein serves to mask and stabilize the hydrophobic, prenylated C-terminus of the GTPase. In fact, the observation that membrane targeting of the Rab family of GTPases is facilitated by an interaction of the Rab protein with Rab escort protein suggests that this postulate is true [32,42]. However, a protein has not yet been identified that serves this escort function for other members of the Ras superfamily. Given the functional importance of GTPase membrane localization, the identification of such escort proteins is a crucial missing element in our understanding of the regulation of cellular events by small GTP-binding proteins.

Although it is possible that GGTase I not only prenylates Rho-family GTPases but also subsequently masks the hydrophobic prenyl group until the GTPase is targeted to a membrane, the present studies directly tested the hypothesis that the interaction of the Rho-family member Cdc42Hs with RhoGDI was an essential intermediate between the prenyltransferase machinery and the acceptor membrane. Our results demonstrate that interaction of Cdc42Hs with RhoGDI is not essential in the targeting of the GTPase to an acceptor membrane but is important in the cycling of Cdc42Hs between the membrane and cytosolic compartments of the cell. Our finding that interaction with RhoGDI is crucial in the regulated intracellular distribution of Cdc42Hs in intact mammalian cells is in agreement with previous observations that RhoGDI extracts Cdc42Hs from membrane preparations in cell-free systems [30,43].

The ability of the GDI-binding defective mutant of Cdc42Hs to stimulate filopodia formation in fibroblasts suggests that interaction with RhoGDI is not necessary to target Cdc42Hs to the appropriate signalling module required for reorganization of the actin cytoskeleton. If membrane localization of Cdc42Hs is important for activation and function of the GTPase, a theory supported by the loss of Cdc42Hs-induced filopodia formation observed in the presence of excess RhoGDI (Figure 8), then our results favour the need for a regulated mechanism by which the interaction of Cdc42Hs with RhoGDI is controlled or disrupted within the normal mammalian cell.

Summary

Taken together, our results support a model (Scheme 1) for the protein–protein interactions that are essential in the regulated intracellular distribution of Cdc42Hs (and probably other Rho family members) in the mammalian cell. Interaction with RhoGDI is not an essential part of the mechanism by which Cdc42Hs is initially targeted to acceptor membranes. However, RhoGDI does seem to have a role in retaining Cdc42Hs in the cytosol and preventing its association with membranes or, alternatively, it is important in the regulated ‘solubilization’ or redistribution of Cdc42Hs from membrane pools. If RhoGDI does function to retain Cdc42Hs in the cytosol and prevent it from reaching an acceptor membrane, we predict that another signal or protein must stimulate the regulated release of Cdc42Hs from RhoGDI and permit Cdc42Hs to be delivered to the membrane for activation. Finally, the mechanism by which the translocation of newly prenylated Cdc42Hs to the membrane

Scheme 1 Model for the regulated intracellular distribution of Cdc42Hs in mammalian cells

Our results demonstrate that the interaction of nascent prenylated Cdc42Hs with RhoGDI is not a prerequisite step in the delivery of this GTPase to the membrane fraction (steps 1A and 1B). However, RhoGDI does seem to have a crucial role in regulating the subsequent distribution of Cdc42Hs between the membrane and cytosolic fractions and is essential in the redistribution of Cdc42Hs from the membrane fraction back into the cytosol (step 2). The question remains as to how newly prenylated Cdc42Hs is shuttled from the cytosolic prenyltransferase complex to the membrane fraction (step 3).

fraction is accomplished is still unresolved. The possibility remains that an as yet unidentified or unconsidered Cdc42Hs interacting protein might fulfil this escort function. Extensive characterization *in vivo* and knockout of protein–protein interactions coupled with Cdc42Hs localization studies will be required to address this question.

We thank L. E. Limbird for critical reading of the manuscript, and E. G. Delfosse for the design and manufacture of the filter punch used to cut filter discs for the GAP assays. This work was supported in part by funding from the American Cancer Society, IRG-91-022-06 and RPG-00-213-01-CCG.

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Received 12 June 2001; accepted 1 August 2001