An activating mutant of Rac1 that fails to interact with Rho GDP-dissociation inhibitor stimulates membrane ruffling in mammalian cells

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Rac1, a member of the Rho family of small GTP-binding proteins, is involved in the regulation of the actin cytoskeleton via activation of lamellipodia and membrane ruffle formation. RhoGDI (Rho-family-specific GDP-dissociation inhibitor) forms a complex with Rho proteins in the cytosol of mammalian cells. It not only regulates guanine nucleotide binding to Rho proteins, but may also function as a molecular shuttle to carry Rho proteins from an inactive cytosolic pool to the membrane for activation. These studies tested if RhoGDI is necessary for the translocation of Rac1 from the cytosol to the plasma membrane for the formation of membrane ruffles. We describe a novel mutant of Rac1, R66E ($Arg^{66} \rightarrow Glu$), that fails to bind RhoGDI. This RhoGDI-binding-defective mutation is combined with a Rac1-activating mutation G12V, resulting in a double-mutant [Rac1(G12V/R66E)] that fails to interact with RhoGDI in COS-7 cells, but remains constitutively activated. This double mutant stimulates membrane ruffling to a similar extent as that observed after epidermal growth factor treatment of non-transfected cells. To confirm that Rac1 can signal ruffle formation in the absence of interaction with RhoGDI, Rac1(G12V) was overexpressed in cultured mesangial cells derived from a RhoGDI knockout mouse. Rac1-mediated membrane ruffling was indistinguishable between the RhoGDI(−/−) and RhoGDI(+/+) cell lines. In both the COS-7 and cultured mesangial cells, Rac1(G12V) and Rac1(G12V/R66E) co-localize with membrane ruffles. These findings suggest that interaction with RhoGDI is not essential in the mechanism by which Rac1 translocates to the plasma membrane to stimulate ruffle formation.

Key words: cytoskeleton, localization, Rac1, RhoGDI (Rho-family-specific GDP-dissociation inhibitor), targeting.

INTRODUCTION

Rac1, a member of the Rho family of small GTP-binding proteins, functions as a molecular switch to regulate a variety of biological processes, including the regulation of the actin cytoskeleton through formation of lamellipodia and membrane ruffles [1]. Rac1 also regulates phagocytosis, superoxide production, endocytosis, secretion, apoptosis, transcriptional activation, cell-cycle progression and cellular transformation [2–8]. The ability of Rac1, similar to other small G-proteins, to interact with effector molecules is dependent on the guanine nucleotide binding state of the GTPase. There are three classes of proteins that control the guanine nucleotide binding state, including GEFs (guanine nucleotide exchange factors) that catalyse the GDP–GTP exchange reaction to activate GTP-binding proteins, GAPs (GTPase-activating proteins) that enhance the intrinsic GTPase activity of these proteins and GDIs (GDP-dissociation inhibitors) that stabilize the inactive GDP-bound form of these GTPases [9]. There is evidence that interaction with GEFs may be one way to provide specificity of signalling by small GTPases [10]. Another mechanism to provide specificity of signalling is probably by the regulation of subcellular localization.

RhoGDI (Rho-family-specific GDI) plays a role in regulating the subcellular localization of Rho proteins. For example, RhoGDI stabilizes the GDP-bound form of Rho proteins within the cytosolic compartment [11,12]. Membrane localization, however, is crucial for coupling many GTPases with their downstream effectors [13,14]. Consequently, Rho proteins undergo a change in intracellular localization from the cytosol to the membrane on activation of the GTP-binding and hydrolysis cycle [15–19]. In addition to maintaining an inactive cytosolic pool of Rho proteins, two additional functions of RhoGDI include the inhibition of GTP hydrolysis and the ability to extract Rho proteins from lipid bilayers, thereby controlling the balance between the inactive cytosolic pool and the active membrane-bound pool [20–22]. These activities of RhoGDI raise the possibility that this regulatory protein may serve as an escort to shuttle Rho proteins to membrane-associated signalling complexes, from their inactive cytosolic pool, on stimulation. It has been shown, for example, that Rac1 dissociates from RhoGDI to translocate from the cytosol to the membrane on stimulation; however, the direct role of RhoGDI in this translocation process has not been fully determined [23–27]. The Rac1–RhoGDI complex is essential for Rac1-mediated regulation of the NADPH oxidase system in neutrophils and recent work by Cerione and co-workers [28] demonstrates that RhoGDI is essential for Cdc42-mediated cellular transformation, suggesting that RhoGDI may carry Rho proteins to important signal-transduction cascades in mammalian cells. In the present study, the possibility that RhoGDI functions as an escort protein to deliver Rac1 to membrane-associated signalling complexes that

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; FBS, foetal bovine serum; GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GEF, guanine nucleotide exchange factor; [^{3}H]GG, tritiated geranylgeranyl; GGTaseI, geranylgeranyl transferase I; GST, glutathione S-transferase; PAK, p21-activated kinase; PBD, protein-binding domain; RhoGDI, Rho-family-specific GDI; WT, wild-type.

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are necessary for reorganization of the actin cytoskeleton into membrane ruffles is addressed.

These studies characterize a novel mutant of Rac1, R66E ($Arg^{66} \rightarrow Glu$), that fails to bind RhoGDI. The G12V amino acid substitution is also utilized to obtain a constitutively activated Rac1 phenotype that can induce membrane ruffling in the absence of stimulation [29]. Coupling the RhoGDI-binding defective mutant with the constitutively activated mutant of Rac1 produced a double-mutant Rac1(G12V/R66E), which was capable of producing membrane ruffles in COS-7 cells to an extent similar to that observed after EGF (epidermal growth factor) treatment of non-transfected cells. To confirm that Rac1 can signal ruffle formation in the absence of interaction with RhoGDI, Rac1(G12V) was overexpressed in cultured mesangial cells derived from a RhoGDI knockout mouse. Rac1-mediated membrane ruffling was indistinguishable between the RhoGDI(−/−) and RhoGDI(+/+) cell lines. In both the COS-7 and cultured mesangial cells, Rac1(G12V) and Rac1(G12V/R66E) co-localize with membrane ruffles. These findings suggest that RhoGDI is not essential for localization of Rac1 to the plasma membrane where Rac1 facilitates membrane-ruffle formation.

EXPERIMENTAL

Epitope tagging and mutagenesis

cDNAs encoding Rac1 and RhoGDIα were modified by the addition of N-terminal epitope tags (for Rac1, myc tag was EQKLISEEDL; for RhoGDI, FLAG tag was DYKDDDDK) by PCR with Deep Vent Polymerase (New England Biolabs, Beverly, MA, U.S.A.) using pCMV5Rac1 and pCMV5RhoGDI respectively as templates. Point mutations were introduced into the coding sequence of human Rac1 by overlap-extension PCR using pCMV5mycRac1 as the template DNA [30]. The DNA sequence of all constructs was verified by DNA sequencing (Cleveland Genomics, Cleveland, OH, U.S.A.). Constructs were subcloned into pET17b for expression as recombinant proteins and into pRK5 for expression in mammalian cells.

In vitro co-precipitation of mycRac1–FLAGRhoGDI complex

Recombinant proteins of mycRac1 and FLAGRhoGDI were produced by the induction of BL21(DE3)pLysS *Escherichia coli* with 1 mM isopropyl β-D-thiogalactoside as described previously [31]. mycRac1 and FLAGRhoGDI were co-precipitated as described previously [22]. Briefly, approximately equal amounts of prenylated mycRac1 WT (wild-type) and mutant recombinant proteins were mixed with approx. 7 μg of recombinant FLAGRhoGDI. The mixtures were then incubated with anti-FLAG affinity gel (Sigma) and the complexes were isolated by centrifugation. The beads were then washed three times with phosphate wash buffer (10 mM $NaPO_4$/1 mM $MgCl_2$/200 μM GDP/0.15 M NaCl) and eluted with 1.0 M glycine (pH 3.0). The eluted complexes were neutralized immediately by the addition of 1 M Tris (pH 8.0). The samples were mixed with SDS sample buffer and analysed by SDS/PAGE and fluorography [32]. Gel bands containing [^{3}H]GG (tritiated geranylgeranyl) mycRac1 were cut from the gels and these were solubilized by incubation for 16 h at 65 °C in 30 % (w/v) H_2O_2 and quantified by scintillation counting.

Intact cell co-precipitation of the mycRac1–FLAGRhoGDI complex

Approx. 20 h before transfection, COS-7 cells were plated at a density of 4.0×10^5 cells/100 mm dish in growth media [DMEM (Dulbecco's modified Eagle's medium)/10 % (v/v) FBS (foetal bovine serum)]. Cells were transfected using combinations of DNA containing 6 μg of either pRK5 or FLAGRhoGDI plus 6 μg of either pRK5mycRac1(WT), pRK5mycRac1(R66E), pRK5mycRac1(G12V) or pRK5mycRac1(G12V/R66E). Transfections were performed with FuGENE6 (Roche, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions using a 2:3 DNA/FuGENE6 ratio. Isolation of the cytosol and subsequent precipitation of mycRac1–FLAGRhoGDI complexes as well as subcellular fractionation and Western-blot analysis were performed as described previously [22]. Antibodies used in Western-blot analyses included the following: anti-myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) as primary antibody and [^{125}I]IgG goat anti-mouse (PerkinElmer) as secondary antibody for Rac1 expression and anti-RhoGDIα (A-20; Santa Cruz Biotechnology) as primary antibody and [^{125}I]IgG goat anti-rabbit (PerkinElmer) as secondary antibody for RhoGDI expression.

Rac1 activation assay

Activation of Rac1 was assayed by co-precipitation of mycRac1 with the GST (glutathione S-transferase)-tagged Rac1-binding domain of PAK (p21-activated kinase) essentially as described previously [33]. Briefly, mycRac1 constructs were overexpressed in COS-7 cells by transfection with FuGENE6 as described above. At approx. 18 h after transfection, cells were harvested by scraping into Dulbecco's PBS and pelleted by centrifugation. Pelleted cells were solubilized in buffer C [25 mM Tris/HCl, pH 7.5/1 mM DTT (dithiothreitol)/30 mM $MgCl_2$/40 mM NaCl/1 % Nonidet P40] and spun to remove the insoluble material. The supernatant was incubated with the Rac1-binding domain of PAK (PAK-PBD, where PBD stands for protein-binding domain) that was fused to GST and bound to glutathione–agarose beads. Binding of PAK-PBD was accomplished by incubation of glutathione–agarose beads with crude recombinant GST-tagged PAK-PBD in the presence of buffer A (25 mM Tris/HCl, pH 7.5/1 mM DTT/30 mM $MgCl_2$/40 mM NaCl) with rotation at 4 °C. The resin was washed with buffer B (25 mM Tris/HCl, pH 7.5/1 mM DTT/30 mM $MgCl_2$/40 mM NaCl/0.5 % Nonidet P40) and buffer A before adding the COS-7 cell lysate. Loaded glutathione–agarose was incubated with the COS-7 cell lysate by rotation. The resin was then washed with buffers B and A. mycRac1 co-precipitated with PAK-PBD was eluted by boiling the resin with SDS sample buffer. Samples were separated by SDS/PAGE (12.5 % gel) and transferred on to PVDF for subsequent Western-blot analysis. Anti-myc antibody (9E10; Santa Cruz Biotechnology) was used as the primary antibody and [^{125}I]IgG goat anti-mouse (PerkinElmer) as secondary antibody.

Immunofluorescence of COS-7 cells

COS-7 cells were plated at a density of 2.5×10^5 cells/100 mm dish, each dish containing uncoated 1.8 cm round coverslips in growth media [DMEM/10 % (v/v) FBS]. Approx. 20 h after plating, cells were serum-starved by replacement of the growth media with DMEM/0.5 % FBS for 18 h. For EGF stimulation, 500 ng/ml EGF (Invitrogen, Carlsbad, CA, U.S.A.) was added to non-transfected, serum-starved cells for 1 h before the coverslips were removed and stained for actin as described previously [22]. For transfection studies, the cells were transfected with FuGENE6 using 12 μg of either pRK5, pRK5mycRac1(WT), pRK5mycRac1(G12V), pRK5mycRac1(R66E) or pRK5mycRac1(G12V/R66E) as described above. Approx. 18 h after

transfection, the coverslips were removed and stained for actin and myc as described previously [22]. Actin was stained with rhodamine–phalloidin (cytoskeleton) and myc was detected using anti-myc antibody (9E10; Santa Cruz Biotechnology) as the primary antibody and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, U.S.A.) as the secondary antibody. Slides were viewed and photographed on a Zeiss Axioplan 2 fluorescence microscope. Four categories of membrane ruffling were determined from the phenotypes observed and populations of cells were scored based on these categories. One category of cells had dramatic ribbon-like ruffling along the cell periphery with sharp co-localization of the mycRac1 construct to these sites of ruffling. Another category of cells also demonstrated membrane ruffling and co-localization of the GTPase to these ruffles, but the ruffling was less significant compared with that seen in cells with the ribbon-like ruffling and was more characteristic of the phenotype seen with EGF stimulation. In another category of cells, mycRac1 constructs localized to the cell periphery, but very little to no ruffling was observed. In the final category of cells, there was no obvious change in the organization of the actin cytoskeleton compared with vector-alone-transfected cells. Cells were scored as having nuclear staining of mycRac1 constructs if most of the protein localized to the nucleus. An average of 300 cells for each transfection condition was scored.

Generation of primary RhoGDI knockout cells

Mouse mesangial cells were isolated by the method of Sage and co-workers [34]. Six-week-old transgenic mice lacking RhoGDIα(−/−) or WT mice (C57Bl/6J) were killed with diethyl ether, the kidneys removed, the cortex dissected, diced and passed through a 212 μm sieve. The resulting homogenate was passed over a 150 μm sieve and a crude glomerular fraction was collected on a 40 μm sieve. The fraction was digested with 2.5 % (v/v) collagenase (CLS4; Worthington Biochemical, Lakewood, NJ, U.S.A.) for 10 min at 37 °C and the glomeruli were sedimented by gravity for 5 min, resuspended in PBS containing 5 % (v/v) calf serum and subjected to microdissection. A highly purified population of glomeruli was plated on 35 mm tissue culture dishes, coated overnight with fibronectin (2 μg/mm^2). Cells were cultured for 10 days in culture media consisting of RPMI 1640, containing penicillin, streptomycin and fungizone and supplemented with 10 mm Hepes, 8 % FBS, 8 % (v/v) bovine calf serum (Hyclone, Logan, UT, U.S.A.), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium plus mouse EGF at 10 ng/ml. At this point, mesangial cell outgrowth was observed and cells were examined for specific cell-marker proteins. In both transgenic and WT cultures, more than 95 % of the cells stained for the mesangial-cell-specific markers α-smooth-muscle actin, desmin and vimentin.

To characterize the expression of RhoGDI isoforms, total protein extracts were prepared from C57BL/6J mouse brain, U373 (human astrocytoma) cells, Jurkat (human T lymphocyte) cells and mouse mesangial cells cultured from WT (+/+) and RhoGDIα knockout (−/−) mice. To prepare the mouse brain extract, freshly dissected forebrain and cerebellum were immediately frozen, ground and extracted for 30 min at 4 °C using solubilization buffer containing 150 mM NaCl, 50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 1 % Triton X-100 and 0.5 % sodium deoxycholate and supplemented with protease inhibitors, PMSF, aprotinin, leupeptin and pepstatin A. Cultured cells were harvested in PBS with protease inhibitors, pelleted and resuspended in solubilization buffer. After extraction, samples were clarified by centrifugation and 20 μg of protein was resolved on a 4–20 % (w/v) polyacrylamide gel and transferred on to PVDF membranes. Membranes were blocked with 5 % (w/v) non-fat milk in PBS containing 0.2 % Tween 20 and incubated overnight with primary antibodies raised against RhoGDIα (sc 360; Santa Cruz Biotechnology) or RhoGDIβ (sc 11359; Santa Cruz Biotechnology). The RhoGDIβ antibody also recognizes RhoGDIγ to a small degree (manufacturer's product information). Primary antibodies were detected using horseradish peroxidase-conjugated Protein A (Sigma) and visualized using a chemiluminescence reagent from PerkinElmer (Western Lightning). After stripping, blots were reprobed with an anti-β-tubulin antibody (sc 9104; Santa Cruz Biotechnology).

Immunofluorescence of mesangial cells

Mesangial cells, both WT and RhoGDI knockout, were plated at a density of 25 000 cells per 35 mm dish with one uncoated 1.8 cm round coverslip in growth media (RPMI 1640 with penicillin and streptomycin, supplemented with 8 % FBS, 8 % calf serum, 10 mm Hepes, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium). At approx. 20 h after being plated, cells were serum-starved by replacement of growth media with RPMI 1640/0.5 % FBS for 18 h. The cells were then either treated with 500 ng/ml EGF (Invitrogen) for 1 h before the coverslips were removed and stained for actin and myc as described previously or transfected with 2.5 μg of pRK5 or pRK5mycRac1(G12V) using LIPOFECTAMINE™ Plus (Invitrogen) according to the manufacturer's instructions with 6 μl of Plus and 4 μl of LIPOFECTAMINE™ [46]. At approx. 18 h after transfection, the coverslips were removed and processed for actin and myc detection [22]. Actin was stained with rhodamine–phalloidin (cytoskeleton) and myc was detected with anti-myc antibody (9E10; Santa Cruz Biotechnology) as the primary antibody and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) as the secondary antibody. Slides were viewed and photographed on a Zeiss Axioplan 2 fluorescence microscope. Investigators determined four categories of membrane ruffling from the phenotypes observed and scored populations of cells based on these categories. These categories are distinct from those observed in COS-7 cells. One category of cells had membrane ruffling along the cell periphery with co-localization of mycRac1(G12V) to these sites of ruffling. Another category of cells had a reorganization of the vast network of actin filaments out to the periphery of the cell into a cortical actin cytoskeletal arrangement. These cells did not exhibit ruffling and were characteristic of the phenotype seen with EGF stimulation. In another category of cells, there was a minor reorganization of the actin cytoskeleton in which the actin filaments were beginning to move towards the periphery of the cell. In the final category, cells had no obvious change in the organization of the actin cytoskeleton compared with vector-alone-transfected cells. An average of 100 cells from each of the five independent experiments was scored.

RESULTS

mycRac1(R66E) and mycRac1(G12V/R66E) display diminished interaction with FLAGRhoGDI both *in vitro* and in intact cell systems

To understand the role played by RhoGDI in regulating the subcellular localization and function of Rac1, we constructed a mutant of Rac1 that was incapable of binding to RhoGDI. We previously identified Arg-66 as being crucially involved in the interaction of Cdc42 with RhoGDI [22]. Crystal structure data

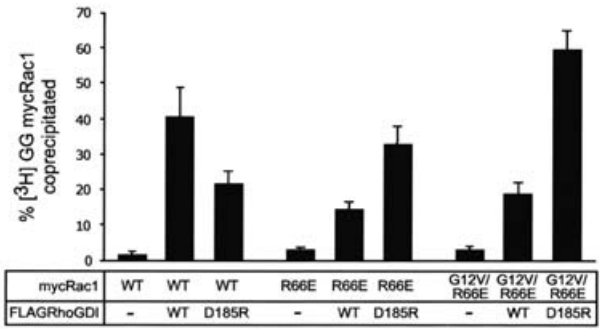

Figure 1 ***In vitro*** **co-precipitation of mycRac1(R66E) and mycRac1(G12V/R66E) with FLAGRhoGDI**

Equal amounts of prenylated mycRac1 WT, R66E and G12V/R66E mutant recombinant proteins were assayed for RhoGDI interaction by the *in vitro* co-precipitation assay as described in the Experimental section. Results are expressed as the percentage of prenylated mycRac1 co-precipitated with RhoGDI(WT) or RhoGDI(D185R). Error bars represent S.E.M. for four independent experiments.

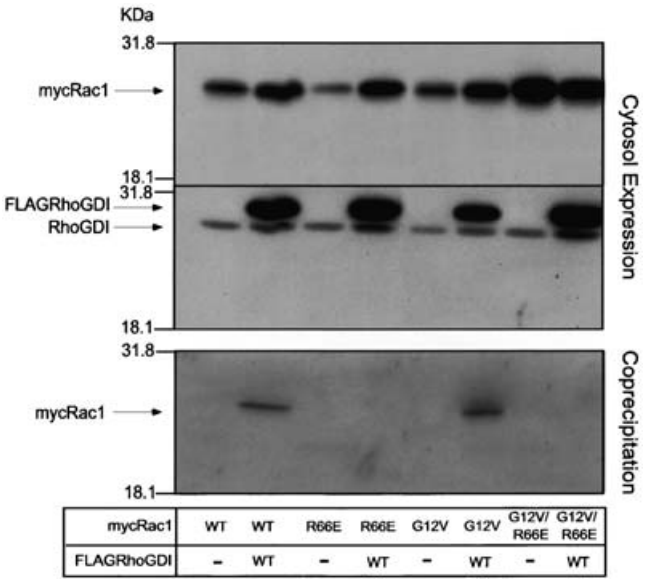

Figure 2 Co-precipitation of mycRac1(R66E) and mycRac1(G12V/R66E) with FLAGRhoGDI in COS-7 cells

Overexpressed mycRac1 WT, R66E, G12V and G12V/R66E mutant proteins were assayed for RhoGDI interaction in COS-7 cells by the intact cell co-precipitation assay as described in the Experimental section. Western-blot analysis was performed using anti-myc or anti-RhoGDI antibody as the primary antibody and [^{125}I]IgG goat anti-mouse or anti-rabbit antibody respectively as the secondary antibody. The cytosol expression autoradiographs (top and middle panels) were exposed for 70 h and represent 25 % of the total cytosolic fraction. The co-precipitation autoradiograph (bottom panel) was exposed for 7 days and represents the entire co-precipitated fraction. Autoradiographs shown are representative of three independent experiments.

of the Cdc42–RhoGDI complex support the involvement of this residue in a direct interaction with RhoGDI [35]. Cdc42(R66E) fails to interact with RhoGDI, but is otherwise normal with respect to prenylation, guanine nucleotide binding and GAP-mediated hydrolysis of GTP [22]. Furthermore, Cerione and co-workers [28] have demonstrated recently that the R66A mutant of Cdc42 is defective with regard to RhoGDI interaction, but is still competent to bind effectors such as PAK, ACK (activated Cdc42-associated tyrosine kinase), WASP (Wiscott–Aldrich syndrome protein), IQGAP (IQ motif containing GTPase activating protein) and the γ-coatomer subunit of the coatomer 1 complex [28]. On the basis of these observations and analysis of the crystal structure of Rac1 complexed with RhoGDI [36], we altered the Arg-66 of human Rac1 to a glutamic residue in an attempt to disrupt Rac1–RhoGDI interaction.

Combining the RhoGDI-binding-defective mutation (R66E) of Rac1 with an activating mutation (G12V) that typically induces ruffle formation in mammalian cells enables the determination of the importance of the Rac1–RhoGDI interaction in Rac1-mediated membrane ruffling [29,37]. Rac1(R66E) and Rac1(G12V/R66E) constructs were assayed for their ability to bind to RhoGDI utilizing an *in vitro* co-precipitation assay (Figure 1) [22]. Since RhoGDI binds to Rho proteins in a prenylation-dependent manner [38], the *in vitro* co-precipitation assay was performed by first prenylating recombinant mycRac1 proteins and then combining equal amounts of prenylated GTPase with recombinant FLAG-tagged RhoGDI. The ability of mycRac1(R66E) and mycRac1(G12V/R66E) to be prenylated indistinguishably from the WT protein (results not shown) is one indication that the introduction of the R66E mutation does not dramatically alter the tertiary structure of the GTPase. *In vitro* binding of recombinant mycRac1 to recombinant FLAGRhoGDI(WT) was dramatically decreased by the introduction of the R66E mutation when compared with WT mycRac1, whereas the mycRac1(G12V/R66E) double mutant also showed diminished interaction with FLAGRhoGDI(WT) (Figure 1).

From the structural data describing the Rac1–RhoGDI complex [36], it can be seen that Arg-66 of Rac1 forms a hydrogen bond with Asp-185 of RhoGDI. It appears that the charge repulsion created by the R66E mutation of Rac1, with Asp-185 of RhoGDI, inhibits complex formation. To support the theory that the Rac1–RhoGDI interaction is disrupted by the introduction of the R66E mutation, the Asp-185 of RhoGDI was mutated to an arginine residue. This 'rescue' mutation in RhoGDI, D185R, was predicted to restore charge attraction with Rac1(R66E). This novel rescue mutant, FLAGRhoGDI(D185R), restored interaction with both mycRac1(R66E) and mycRac1(G12V/R66E) in the *in vitro* co-precipitation assay to a level statistically similar to the original WT Rac1–RhoGDI complex (Figure 1).

The inability of the R66E-containing constructs of Rac1 to interact with RhoGDI was also assayed in intact cells. mycRac1 constructs were overexpressed in COS-7 cells and their ability to co-precipitate with overexpressed FLAGRhoGDI was assessed by a co-precipitation assay (Figure 2) [22]. As seen in the co-precipitation autoradiograph in Figure 2, both mycRac1(WT) and the activating mutant mycRac1(G12V) interact with RhoGDI in the cytosolic fraction of overexpressing COS-7 cells, whereas the R66E mutation appears to prevent completely interaction of mycRac1 with FLAGRhoGDI. The data from both the *in vitro* and intact cell co-precipitation assays demonstrate that the addition of the R66E mutation to the constitutively activated Rac1, G12V, disrupts the ability of this construct [Rac1(G12V/R66E)] to interact with RhoGDI.

FLAGRhoGDI does not solubilize mycRac1(R66E) or mycRac1(G12V/R66E) from membranes of COS-7 cells

One function of RhoGDI is to extract Rho proteins from lipid bilayers [22,39]. The ability of RhoGDI to interact functionally with Rac1 by solubilizing the GTPase from membranes was assessed by the subcellular fractionation of mycRac1 constructs overexpressed in COS-7 cells in the presence of overexpressed FLAGRhoGDI (Figure 3). Co-overexpression of FLAGRhoGDI with WT mycRac1 dramatically shifts the localization of the GTPase from the membrane to the cytosolic fraction of

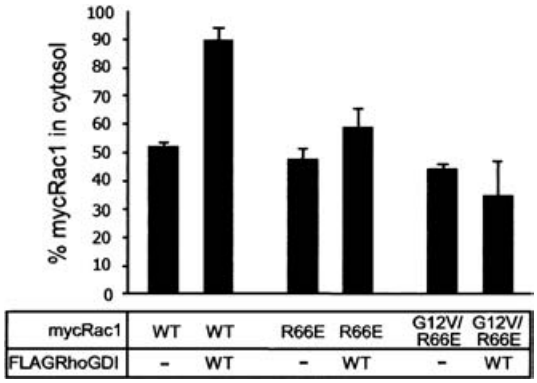

Figure 3 Solubilization of mycRac1(R66E) and mycRac1(G12V/R66E) from membranes of COS-7 cells with overexpressed FLAGRhoGDI

mycRac1 WT, R66E and G12V/R66E mutant proteins were overexpressed along with FLAGRhoGDI in COS-7 cells. Microsomal membranes were separated from the cytosol by the subcellular fractionation assay as described in the Experimental section. Data are expressed as the percentage of total mycRac1 that localized to the cytosolic fraction. Error bars represent S.E.M. for three independent experiments.

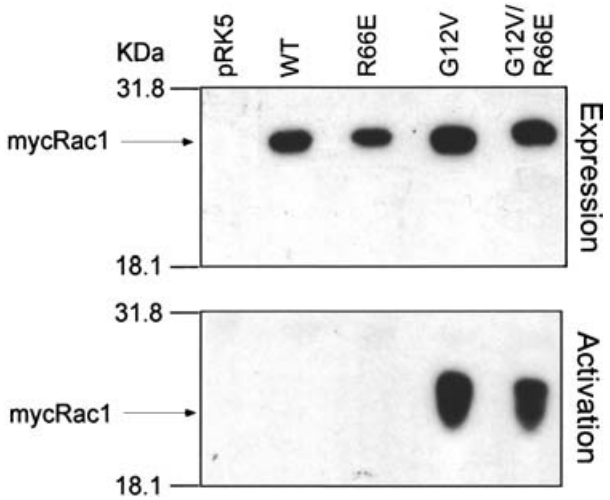

Figure 4 Activation of mycRac1(G12V/R66E)

The activation states of mycRac1 WT, R66E, G12V and G12V/R66E were assessed by co-precipitating COS-7 cell lysates overexpressing mycRac1 constructs with glutathione–agarose and the GST-fused Rac1-binding domain of PAK. Western-blot analysis was performed using anti-myc antibody as the primary antibody and [^{125}I]IgG goat anti-mouse antibody as the secondary antibody. pRK5 was used as a vector control. The expression autoradiograph (upper panel) represents the total protein overexpressed and the activation autoradiograph (lower panel) represents the GTP-bound GTPase eluted from the activation assay as described in the Experimental section. Both autoradiographs were exposed for 4 days. Autoradiographs shown are representative of two independent experiments.

COS-7 cells. However, co-overexpression of FLAGRhoGDI with mycRac1(R66E) or mycRac1(G12V/R66E) only very minimally, if at all, affects the localization of these GTPase constructs, further confirming the inability of the R66E-containing constructs of Rac1 to interact functionally with RhoGDI.

mycRac1(G12V/R66E) is activated to a similar extent as mycRac1(G12V)

Before utilizing the Rac1(G12V/R66E) double mutant in functional studies, it was critical to determine first that the introduction of the R66E mutation into Rac1(G12V) did not alter the constitutively activated state of this mutant. Binding of mycRac1 to PAK-PBD, an effector of Rac1, was utilized to assess the activation of mycRac1 constructs expressed in COS-7 cells (Figure 4) [33]. The activation of the double mutant was comparable with that of the G12V single mutant. This confirms that addition of the R66E mutation did not affect the ability of this protein to bind GTP. Guanine nucleotide binding to Rac1(G12V/R66E) provides further evidence that this protein retains a functional tertiary structure. Demonstrating that Rac1(G12V/R66E) is activated to the same extent as Rac1(G12V) was critical to the next goal, namely to assess the functional importance of preventing the activating mutant from interacting with RhoGDI.

Constitutively activated Rac1 can stimulate membrane ruffling in COS-7 cells in the absence of interaction with RhoGDI

The RhoGDI-binding-defective mutant of Rac1, R66E, both alone and in combination with the activating mutant G12V/R66E, was used to determine the potential role of RhoGDI as an escort protein to translocate Rac1 to membrane-associated signalling complexes necessary for ruffle formation. The constitutively activated mutant, G12V, was utilized to selectively activate overexpressed Rac1 rather than using a physiological stimulator of Rac1, such as EGF, which can activate not only endogenous Rac1 that can interact with RhoGDI but also overexpressed R66E-containing mycRac1 constructs that cannot interact with RhoGDI. The results in Figure 5 depict immunofluorescence imaging of overexpressed mycRac1 constructs in COS-7 cells. Figure 5(A) shows the extent of ruffle formation induced by a physiological activator of Rac1, namely EGF, in non-transfected COS-7 cells. After the addition of EGF, a reorganization of the actin cytoskeleton into membrane ruffles at the cell periphery can be seen. Transfection with the vector control alone produced no response from the actin cytoskeleton (Figure 5B).

The most extensive membrane ruffling (Figures 5C and 5E) produced a ribbon-like organization of the actin cytoskeleton along the cell periphery, with very sharp co-localization of mycRac1 to these sites of ruffling. Most of the mycRac1(G12V)-overexpressing cells (72%) produced this extensive ribbon-like ruffling, whereas only a small number of mycRac1(G12V/R66E)-overexpressing cells (8%) were characterized by this morphological phenotype. The majority of mycRac1(G12V/R66E)-overexpressing cells (90%, Figure 5F) and a lesser number of the mycRac1(G12V)-overexpressing cells (28%, Figure 5D) still produced membrane ruffles, but these ruffles were less significant than the ribbon-like ruffling phenotype seen in Figures 5(C) and 5(E). The ruffling seen in Figures 5(D) and 5(F) is more characteristic of the ruffling phenotype seen with EGF stimulation (Figure 5A). There is co-localization of mycRac1 to the sites of membrane ruffling in the EGF characteristic ruffling pattern (Figures 5D and 5F), but it is more diffuse compared with that seen with the ribbon-like ruffling pattern (Figures 5C and 5E). The ability of mycRac1(G12V/R66E) to stimulate physiological membrane ruffling in the absence of interaction with RhoGDI suggests that RhoGDI is not essential for delivering Rac1 to signalling complexes involved in ruffle formation.

COS-7 cells overexpressing mycRac1(WT) or mycRac1(R66E) did not form readily visible ruffles. The majority of cells overexpressing mycRac1(WT) (79%, Figure 5G) and mycRac1 (R66E) (69%, Figure 5I) displayed localization of mycRac1 at the cell periphery, but very minor to no obvious ruffling was seen. A smaller percentage of cells overexpressing mycRac1(WT) and mycRac1(R66E) (Figures 5H and 5J) resembled vector-alone-transfected cells with no obvious rearrangement of the actin

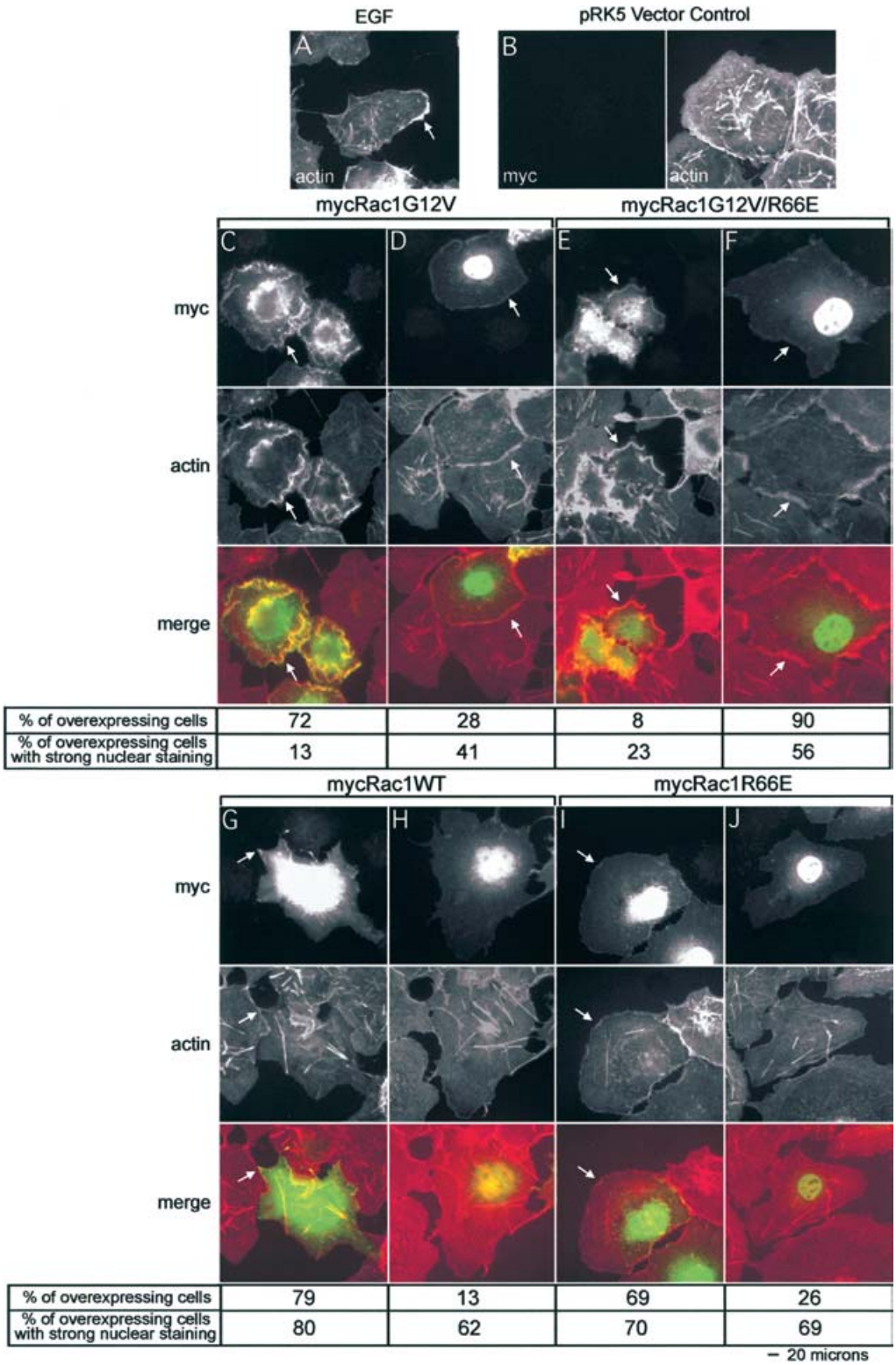

Figure 5 Rac1-mediated membrane ruffling in COS-7 cells

mycRac1 constructs were overexpressed in COS-7 cells. mycRac1 was visualized by staining with anti-myc antibody as the primary antibody and Alexa Fluor 488 goat anti-mouse IgG as the secondary antibody. Actin filaments were labelled with rhodamine–phalloidin. On average, 300 cells per transfection condition were scored, as described in the Experimental section. Quantification data represent the percentage of cells overexpressing the mycRac1 construct having the membrane-ruffling phenotype depicted from the total number of cells counted. Quantification data representing the percentage of cells overexpressing the mycRac1 construct having strong nuclear localization of the GTPase are also given. Arrows show examples of membrane ruffles and co-localization of Rac1 constructs to these ruffles. Bottom panels represent merged images of myc (green) and actin (red). Magnification, × 63. Images shown are representative of cell morphologies seen in three independent experiments.

cytoskeleton or localization of overexpressed mycRac1 to regions of the cell periphery. Finally, different extents of ruffling (ribbon-like, EGF-stimulated-like, very minor and no ruffling) were seen in cells overexpressing each of the four mycRac1 constructs; however, as can be observed from the quantification of the ruffling phenotypes, these phenotypes represent less than 8% of the total overexpressing cells in each case. Therefore only the most predominant phenotypes seen with each construct are depicted in Figure 5. Also, the differences in morphology seen did not relate to protein expression levels, since different degrees of protein expression were seen with each morphological category.

While scoring populations of cells for different ruffling phenotypes, it became apparent that some morphological phenotypes displayed a more intense nuclear localization of the GTPase than others. Interestingly, cells with the extreme ribbon-like ruffling phenotype (Figures 5C and 5E) had very minimal amounts of mycRac1 localized to the nucleus, whereas cells with very minor to no ruffling (Figures 5G and 5I) had almost all of the GTPase localized to the nucleus. Again, this nuclear localization did not appear to be affected by expression levels of mycRac1 constructs. The physiological significance of the nuclear localization of Rac1 is unclear.

RhoGDI is not essential for Rac1-mediated ruffling or localization of mycRac1 to these ruffles in RhoGDI knockout mouse mesangial cells

In the *in vitro* and intact cell studies characterizing the Rac1(G12V/R66E) mutant (Figures 1–3), there is a minimal level of interaction between this double mutant and RhoGDI. An argument could be made that this small amount of interaction with RhoGDI is sufficient to allow for RhoGDI-mediated translocation of Rac1 to the plasma membrane for stimulation of ruffle formation. To confirm that interaction with RhoGDI is not essential in the process by which Rac1 signals ruffling, we developed RhoGDI knockout cells from the RhoGDIα knockout mouse model described previously [40].

The RhoGD1(−/−) cultured mesangial cells are null for RhoGDIα protein expression as demonstrated by Western-blot analysis (Figure 6, top panel). RhoGDIα is ubiquitously expressed [41] and, as shown in Figure 6, is found in mouse brain preparations, U373 cells, Jurkat cells and in the WT mouse mesangial cells, but not in the knockout mouse mesangial cells. There are, however, two other isoforms of RhoGDI (β and γ) that theoretically could substitute for RhoGDIα. It is highly improbable, however, that either of these two proteins plays a role in mesangial cell physiology, since RhoGDIβ is specifically expressed in haematopoietic tissues and the non-cytosolic RhoGDIγ is expressed in brain tissues [42,43]. Nonetheless, expression of these isoforms was considered by Western-blot analysis using an antibody raised against RhoGDIβ (Figure 6, bottom panel). This antibody cross-reacts with RhoGDIγ as evidenced by the strong positive signal obtained from mouse brain and U373 human astrocytoma cell extracts. The band observed in the lane corresponding to mesangial RhoGDI(+/+) in Figure 6 (lower panel) may be the result of slight cross-reactivity of the anti-RhoGDIβ antibody with RhoGDIα or may represent a very minimal expression of the β and γ isoforms of RhoGDI. In any case, the results shown in Figure 6 confirm that the RhoGDI knockout cells are null for all three RhoGDI isoforms.

The activating mutant of Rac1, G12V, was used to confirm that RhoGDI is not necessary for Rac1-induced membrane ruffling. The results in Figure 7 depict immunofluorescence imaging of overexpressed mycRac1(G12V) in both WT (+/+) and RhoGDI knockout (−/−) mesangial cells. Figures 7(A) and 7(G) show the reorganization of the actin cytoskeleton induced by EGF stimulation in non-transfected WT and knockout mesangial cells respectively. EGF treatment induced the same phenotype in both WT and knockout cells, resulting in the reorganization of the network of actin filaments out towards the cell periphery into a cortical cytoskeletal assembly. Transfection with the vector control alone produced no change in the actin cytoskeleton in both the WT and knockout cells (Figures 7B and 7H).

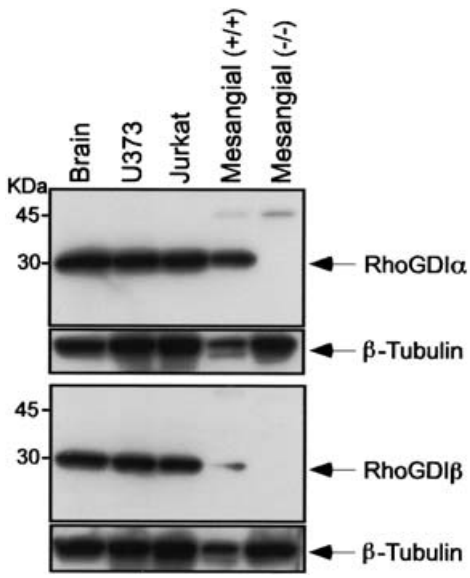

Figure 6 Western-blot analysis of wild-type and RhoGDI knockout mouse mesangial cells

Total protein extracts were prepared from mouse brain, U373 human astrocytoma cells, Jurkat T lymphocytes and mouse mesangial cells cultured from WT (+/+) and RhoGDIα knockout (−/−) mice. Identical membranes were probed using anti-RhoGDIα (top panel) or anti-RhoGDIβ (bottom panel) antibody as the primary antibody and horseradish peroxidase-conjugated Protein A as the secondary antibody and visualized by chemiluminescence. Protein loading was verified by subsequent immunoblotting of the same membranes for β-tubulin.

Although these cells do not exhibit the dramatic ruffling phenotype that can be observed in COS-7 cells, both WT and RhoGDI knockout mesangial cells overexpressing mycRac1(G12V) (Figures 7C and 7I) produced membrane ruffles along the periphery of the cell with co-localization of the GTPase to these sites of ruffling. Between 25 and 30% of WT and knockout overexpressing cells produced this phenotype. A larger percentage of both WT and knockout overexpressing cells (Figures 7D and 7J, 40–43%) produced a reorganization of the actin cytoskeleton into a cortical actin structure characteristic of that seen with EGF stimulation (Figures 7A and 7G). Approx. 20% of both WT and knockout overexpressing cells (Figures 7E and 7K) had a minor alteration in the actin cytoskeleton in which actin filaments appeared to start moving out towards the cell periphery. The remaining approx. 12% of both WT and knockout overexpressing cells (Figures 7F and 7L) resembled vector-alone-transfected cells with no obvious rearrangement of the actin cytoskeleton or localization of the GTPase to regions of the cell periphery. The differences in morphology seen did not relate to protein expression levels, since different degrees of protein expression were seen with each morphological category. Quantification of these various degrees of actin reorganization in mycRac1(G12V)-overexpressing cells (Figure 7) was indistinguishable between WT and RhoGDI knockout cells, confirming that RhoGDI is not essential in delivering Rac1 to signalling complexes necessary

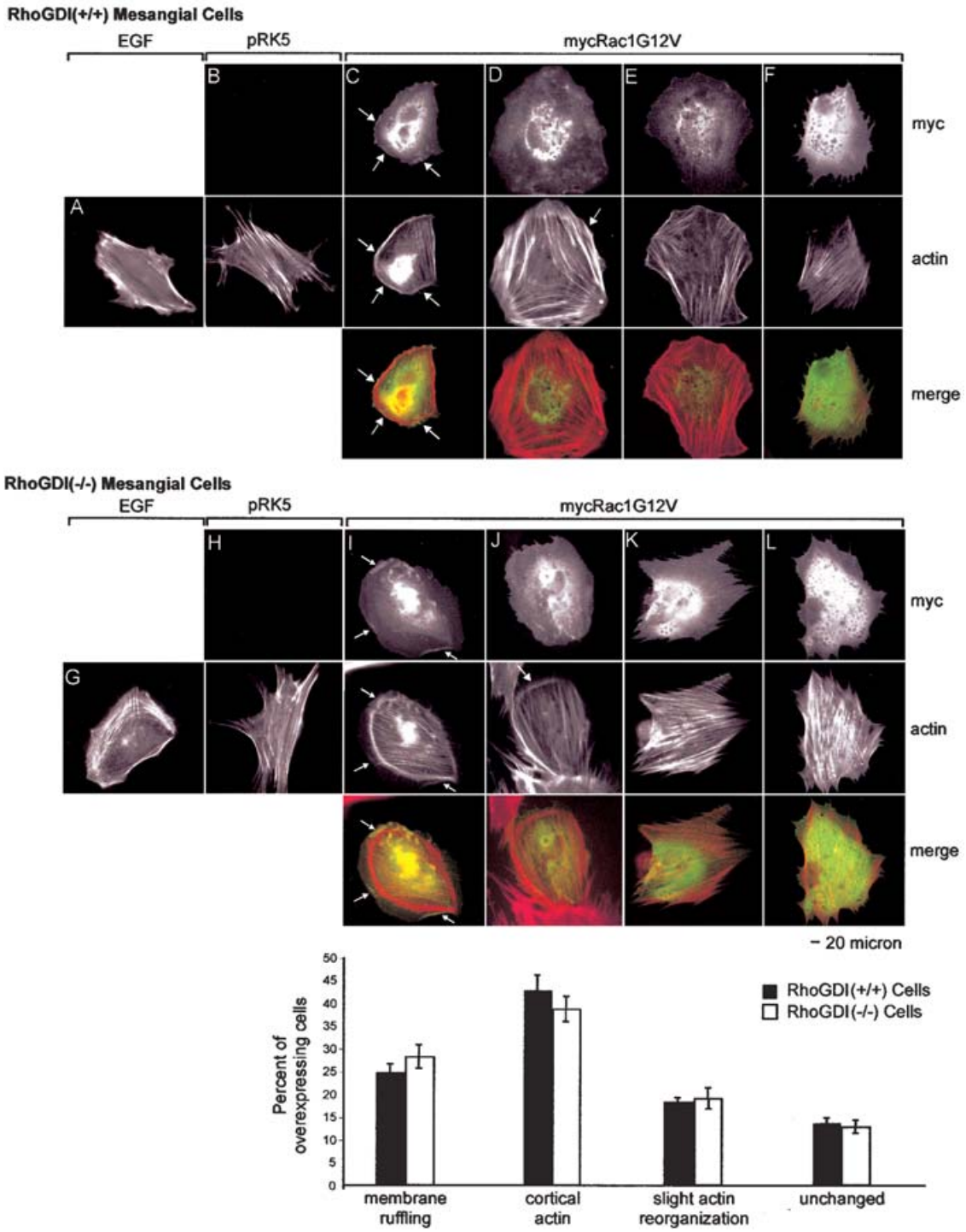

Figure 7 Rac1-mediated membrane ruffling in RhoGDI knockout mouse mesangial cells

mycRac1 constructs were overexpressed in WT and RhoGDI knockout cultured mouse mesangial cells. mycRac1 was visualized by staining with anti-myc antibody as the primary antibody and Alexa Fluor 488 goat anti-mouse IgG as the secondary antibody. Actin filaments were labelled with rhodamine–phalloidin. On average, 100 cells for both WT and knockout cells were scored as described in the Experimental section. The bar graph shows the percentage of cells overexpressing mycRac1(G12V) in WT (black bar) and knockout (open bar) mesangial cells, corresponding to the extent of actin reorganization depicted in the immunofluorescence images above each set of bars. Error bars represent S.E.M. for five independent experiments. Arrows in (**C**, **I**) show examples of membrane ruffles and co-localization of Rac1 constructs to these ruffles. Arrows in (**D**, **J**) show cortical actin structures around the cell periphery. Bottom panels represent merged images of myc (green) and actin (red). Magnification, × 63. The images shown are representative of cell morphologies seen in five independent experiments.

for ruffle formation. Intense nuclear localization of the GTPase, similar to that seen in COS-7 cells overexpressing mycRac1(WT) and mycRac1(R66E), was not observed in WT or RhoGDI knockout cultured mesangial cells.

DISCUSSION

Rho-family GTPases interact with a multitude of effector proteins to regulate diverse signalling pathways [44]. Specificity of

signalling is vital for ensuring a proper physiological response after GTPase activation. Therefore understanding the mechanisms that regulate the diverse and unique signalling pathways of small GTPases is crucial to understand their function. Control of subcellular localization is probably one way to gain specificity of signalling. We have considered the role played by RhoGDI in regulating the subcellular localization and function of the Rho family member Rac1.

RhoGDI functions as a critical regulator of Rho family proteins. By binding to prenylated GDP-bound Rho proteins, RhoGDI is clearly important in maintaining an inactive pool of these GTPases in the cytosol [11,12]. RhoGDI is also capable of solubilizing Rho proteins from lipid bilayers, controlling the balance between the inactive cytosolic pool and the active membrane-bound pool [20,22,39]. Extracting these small GTPases from membranes prevents interaction with GEFs, which are necessary for activation. For example, RhoGDI sequesters Rac1 in the cytosol, blocking integrin-stimulated binding to effectors. However, membrane translocation displaces RhoGDI and allows effectors to bind Rac1 [24]. A recent work of Robbe et al. [23] has also shown that Rac1 dissociation from RhoGDI and its translocation to the membrane are required for the activation of Rac1 by its effector Tiam. Although these studies imply that RhoGDI may deliver Rac1 to signalling complexes at the membrane, we sought to look directly at the interaction between Rac1 and RhoGDI to determine if RhoGDI plays an escort-type function in delivering Rac1 to the plasma membrane for interaction with effectors involved in membrane ruffling. The ability of RhoGDI to regulate guanine nucleotide binding and the intracellular distribution of Rho proteins makes it a probable candidate chaperone protein to facilitate the intracellular targeting of Rac1.

In the present study, a novel RhoGDI-binding-defective mutant of Rac1, R66E, was used to examine specifically the role played by RhoGDI in regulating Rac1-mediated reorganization of the actin cytoskeleton. This RhoGDI-binding-defective mutant was coupled with a constitutively active mutant of Rac1, G12V, to produce a membrane-ruffling phenotype [29,37]. The resulting double-mutant Rac1(G12V/R66E) was used to determine the role of RhoGDI in Rac1-mediated membrane ruffling. The RhoGDI-binding-defective mutant Rac1(R66E), alone and in combination with the activating mutant Rac1(G12V/R66E), displayed substantially diminished interaction with RhoGDI in an *in vitro* co-precipitation assay (Figure 1). The decreased and not completely abolished interaction between R66E-containing constructs of Rac1 and RhoGDI may be due to non-physiological conditions inherent in this and any *in vitro* assay. The significantly decreased interaction of Rac1(R66E) with RhoGDI did, however, identify this mutant as a RhoGDI-binding-defective mutant and encouraged the more physiological characterization of this construct in intact cells. Overexpressed Rac1(R66E) and Rac1(G12V/R66E) failed to interact with overexpressed RhoGDI within the cytosol of COS-7 cells (Figure 2), confirming that, under physiological conditions, the R66E mutation of Rac1 is defective in binding RhoGDI. Subcellular fractionation of COS-7 cells revealed that overexpressed RhoGDI caused a shift in localization of Rac1(WT) from the membrane to the cytosolic fraction (Figure 3). This shift in localization of WT Rac1 is due to the ability of RhoGDI to solubilize Rho proteins from membranes. However, overexpressing RhoGDI did not significantly alter the localization of R66E-containing constructs of Rac1, providing further evidence that Rac1(G12V/R66E) cannot functionally interact with RhoGDI in the cytosol or at the membrane. Additionally, the R66E mutation did not alter the constitutively activated state of Rac1(G12V) (Figure 4). Taken together, the data characterizing Rac1(R66E) show that, analogous to what we have observed for Cdc42 [22], the mutation of Arg-66 within the protein structure of Rac1 disrupts the ability of this GTPase to interact with its negative regulator RhoGDI. Addition of the R66E mutation, however, does not affect the ability of Rac1 to be prenylated (results not shown) or to bind GTP (Figure 4), indicating that this novel mutation of Rac1 does not cause gross structural changes to the protein.

Use of the novel rescue mutant of RhoGDI, D185R, to restore interaction with R66E-containing constructs of Rac1 in the *in vitro* co-precipitation assay (Figure 1) confirmed the mechanism of complex inhibition through the R66E point mutation. Our results are consistent with the theory that this mutation creates charge repulsion with residue 185 of RhoGDI, preventing hydrogen bond formation, thus destabilizing the complex. The rescue mutant RhoGDI(D185R) may prove useful in future studies to restore Rho-protein-mediated functions that require RhoGDI.

Immunofluorescence imaging of overexpressed Rac1(WT) or Rac1(R66E) in COS-7 cells showed little to no ruffling phenotype, making it necessary to utilize an activating mutant of Rac1, G12V, to activate selectively Rac1 and produce a morphological phenotype (Figure 5). Overexpressing the double-mutant Rac1(G12V/R66E) demonstrates that even in the absence of interaction with RhoGDI, an activating mutant of Rac1 is capable of stimulating membrane ruffles and translocating to the plasma membrane (Figure 5). Although the ruffling phenotype seen with overexpressed Rac1(G12V/R66E) was less dramatic compared with the ribbon-like ruffling seen with overexpressed Rac1(G12V), it was similar to the morphological changes seen on EGF stimulation. Overexpressing the activating mutant of Rac1 in RhoGDI knockout mesangial cells that are null for all three isoforms of RhoGDI (α, β and γ) (Figure 6) produced ruffling phenotypes indistinguishable from WT mesangial cells (Figure 7), confirming that RhoGDI is not essential for the delivery of Rac1 to signalling complexes at the plasma membrane responsible for these cytoskeletal rearrangements. The different extents of ruffling seen with overexpressed Rac1(G12V) and Rac1(G12V/R66E) in COS-7 cells may be due to different membrane-associated signalling complexes involved in the different ruffling morphologies observed. For example, perhaps interaction with RhoGDI facilitates the delivery of Rac1 to signalling complexes necessary for the dramatic ribbon-like ruffling observed, whereas interaction with RhoGDI may not be required for the delivery of Rac1 to other signalling complexes necessary for stimulation of EGF-like ruffling. A more straightforward explanation may be that the actual extent of activation of the Rac1(G12V/R66E) mutant is slightly less compared with that observed for Rac1(G12V). Cell type differences may also contribute to the role played by RhoGDI in Rac1-mediated membrane ruffling. In any case, these results do not rule out the possibility that RhoGDI may play a role in translocating Rac1 to effectors involved in other Rac1-mediated functions.

The intense and predominant nuclear localization of Rac1 seen in COS-7 cells with little to no ruffling (Figure 5), compared with the minor nuclear localization of Rac1 seen in cells with more extensive ruffling phenotypes, raises some interesting questions. Nuclear localization of Rac1 has been reported previously; although the function of this pool of GTPase is unknown, it has been shown to be in an inactivated GDP-bound state [45,46]. Williams and co-workers [47] found that the polybasic region at the C-terminus of Rac1 serves as a nuclear localization signal, promoting the association of Rac1 with a guanine nucleotide dissociation stimulator, SmgGDS, and causes the translocation of the SmgGDS–Rac1 complex into the nucleus. In this way, Rac1 may serve to translocate armadillo family proteins such as SmgGDS to the nucleus. The increase in nuclear localization of Rac1 seen

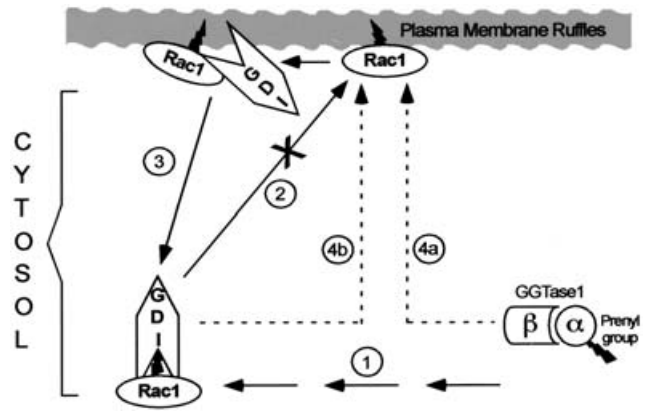

Figure 8 Model for the regulated intracellular distribution of Rac1

Newly prenylated Rac1 is transferred from the cytosolic prenyltransferase machinery to its complex with RhoGDI (step 1) through an as yet unidentified mechanism. RhoGDI is not necessary for translocating Rac1 to signalling complexes at the plasma membrane necessary for membrane-ruffle formation (step 2), but RhoGDI is involved in solubilizing Rac1 from the membrane (step 3). Unidentified protein–protein interactions probably enable the translocation of Rac1 either from the prenyltransferase machinery directly (step 4a) or from RhoGDI (step 4b) to complexes at the membrane responsible for the activation of ruffling. Broken lines indicate possible pathways of localization with accompanying unidentified protein–protein binding intermediates implied.

with decreased reorganization of the actin cytoskeleton (Figure 5) raises the possibility that sequestration of Rac1 in the nucleus prevents activation of Rac1-mediated cytoskeletal rearrangements. The functional significance of this nuclear pool of Rac1 will be considered in future studies.

Figure 8 depicts a model of our current understanding of the regulation of the intracellular distribution of Rac1. Prenylated Rac1 is complexed with RhoGDI in the cytosol. The mechanism whereby newly prenylated Rac1 leaves the cytosolic GGTaseI (geranylgeranyl transferase I) complex to interact with RhoGDI is not known (step 1). The results described in the present study demonstrate that RhoGDI is not required to translocate Rac1 to complexes at the plasma membrane that control reorganization of the actin cytoskeleton into membrane ruffles (step 2). RhoGDI, however, does play a role in extracting Rac1 from the membrane (step 3). Since RhoGDI is not essential for Rac1 localization to the plasma membrane and induction of ruffle formation, it seems probable that there must be some other mechanism whereby Rac1 is translocated from the cytosol to complexes at the plasma membrane necessary for activation of ruffling. There may be as yet uncharacterized protein–protein interactions that facilitate the transfer of Rac1 from either the cytosolic GGTaseI complex (step 4a) or its interaction with RhoGDI (step 4b) to signalling complexes at the plasma membrane involved in membrane ruffling.

Although these studies demonstrate that RhoGDI does not fulfil an escort function in delivering Rac1 to membrane-associated signalling complexes necessary for ruffling, it is important to note that RhoGDI is still a key regulatory protein of small GTPases. For example, the significant phenotypic changes observed in RhoGDI knockout mice, such as kidney and reproductive organ impairment, mark RhoGDI as a critical regulator of Rho family GTPases [40]. *In vivo*, RhoGDI serves as an essential part of the mechanism by which Rho proteins are solubilized from membranes and retained in the cytosol in their inactive, GDP-bound forms. Determining the protein–protein interactions that facilitate the translocation of Rac1 from the cytosol to the plasma membrane will provide insight into additional mechanisms of regulating Rho protein signalling specificity.

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