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A novel role for RhoGDI as an inhibitor of GAP proteins

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RhoGDI inhibits guanine nucleotide dissociation from post-translationally processed Rho and Rac proteins but its biochemical role *in vivo* is unknown. We show here that N-terminal effector site mutations in the Rac protein do not compromise its interaction with RhoGDI and that, whilst geranylgeranylation and -AAX proteolysis of the C-terminal CAAX motif of Rac1 and RhoA are required for efficient interaction with RhoGDI, methylesterification of the C-terminal cysteine residue is not required. *In vitro*, RhoGDI can form stable complexes with Rho and Rac proteins in both the GTP and GDP bound states. Furthermore the Rac-GTP-RhoGDI complex is resistent to the action of recombinant RhoGAP and recombinant BCR. Thus GDI, by complexing with Rac-GTP and preventing GAP stimulated GTP hydrolysis, may allow transit of the activated form of the Rac protein between physically separated activator and effector proteins in the cell.

Key words: GAP/GDI/prenylation/Rac/Rho

Introduction

Ras and Ras-related guanine nucleotide binding proteins cycle between an inactive GDP bound state and an active GTP bound state and regulate a variety of intracellular signal transduction pathways. Most Ras and Ras-related proteins terminate at their C-termini with CAAX, XCC or CXC motifs which undergo post-translational modification. CAAX motifs of Ras proteins are farnesylated (Casey *et al.*, 1989; Hancock *et al.*, 1989) whilst those of all other Ras-related proteins are geranylgeranylated (reviewed in Hancock and Marshall, 1993). Prenylated CAAX motifs are then proteolysed to remove the AAX amino acids and the C-terminal cysteine is carboxylmethylated (Clark *et al.*, 1989; Gutierrez *et al.*, 1989). The XCC and CXC motifs are geranylgeranylated but only the CXC motif is subsequently carboxylmethylated (Khoshari-Far *et al.*, 1991; Kinsella and Maltese, 1992; Newman *et al.*, 1992; Peter *et al.*, 1992).

The activity of Ras-related proteins is controlled by at least two classes of protein. GTPase activating proteins (GAPs) bind to the GTP-bound form and stimulate the intrinsic GTPase activity of the Ras-related protein while guanine nucleotide exchange factors (GEFs) stimulate nucleotide dissociation which *in vivo* leads to the formation of the active GTP bound state. A number of GEFs have been characterized. Ras exchange factors recently cloned from mammals (GRF) (Martegani *et al.*, 1992; Shou *et al.*, 1992) and the fruit fly (Sos) (Simon *et al.*, 1991) show significant homology to the RAS exchange proteins CDC25 and SCD25 of *Saccharomyces cerevisiae* (Broek *et al.*, 1987; Crechet *et al.*, 1990). This family of proteins appears to be specific for the three Ras proteins and to stimulate nucleotide exchange on recombinant proteins lacking post-translational modification. The Dbl oncoprotein functions as an exchange protein for CDC42Hs, a member of the Rho subfamily of Ras-related proteins (Hart *et al.*, 1991) and although a number of proteins having homology to Dbl are now known, including the Vav oncoprotein and Bcr, it is not yet clear if they constitute a family of exchange factors (Hall, 1992a). Takai and co-workers have cloned the gene for a protein smgGDS (Kaibuchi *et al.*, 1991) and defined a further activity termed RhoGDS (Isomura *et al.*, 1990) which regulates nucleotide binding but only on post-translationally processed Ras-related proteins. SmgGDS has no significant homology to CDC25, SCD25 or Dbl but stimulates the release of radiolabelled GDP and GTP from post-translationally processed RhoA, RhoB, Rap1a, Rap1b, Rac1 and K-ras(B). It is inactive on the unmodified, recombinant proteins and also has no activity on H-Ras or N-Ras (Hiroyoshi *et al.*, 1991; Kaibuchi *et al.*, 1991; Mizuno *et al.*, 1991; Hiraoka *et al.*, 1992). The activity of smgGDS on Rap1b can be inhibited by short geranylgeranylated peptides corresponding to the C-terminal sequence of Rap1b but not non-prenylated C-terminal peptides (Shirataki *et al.*, 1991).

Another factor, RhoGDI, was first detected through its ability to inhibit the dissociation of radiolabelled GDP from post-translationally modified RhoA, RhoB and Rac1 (Ueda *et al.*, 1990; Hori *et al.*, 1991; Hiraoka *et al.*, 1992). In addition it has been shown that RhoB when bound to GDP, but not when bound to GTP, can form a stable complex with RhoGDI *in vitro* (Ueda *et al.*, 1990). The *in vivo* role of RhoGDI is still unclear though it is expressed in all cell types so far examined. In addition to inhibiting nucleotide release it affects the interaction of Rho-related proteins with membranes. For example, RhoB is released from membranes on binding RhoGDI (Isomura *et al.*, 1991). Abo *et al.* (1991) showed that a complex of Rac1 and RhoGDI was able to stimulate the superoxide producing NADPH oxidase activity present in neutrophils in an *in vitro* assay. This has raised the possibility that RhoGDI is part of the effector complex in this system and may in fact, contrary to earlier reports, bind to the GTP as well as the GDP form of Rac1. Alternatively RhoGDI complexed with Rac-GTP may facilitate transport of activated Rac1 to the NADPH oxidase complex. We have therefore examined the interaction of RhoA and Rac1 proteins with RhoGDI. We find that RhoGDI forms complexes with both GDP and GTP forms of Rac1 and that the GTP bound complex is resistent to GAP proteins.

Results

Expression of RhoGDI and post-translationally modified RhoA and Rac1

Human RhoGDI was obtained by PCR cloning from human lymphocyte cDNA using the bovine RhoGDI sequence to design appropriate PCR primers (see Materials and methods). The human cDNA sequence predicts a protein of 204 amino acids, the same length as bovine RhoGDI with which it is highly homologous having seven conservative amino acid substitutions outside of the PCR primer sequences. Human RhoGDI was expressed in *Escherichia coli* as a glutathione-S-transferase (GST) fusion protein and purified on glutathione agarose beads. The preparations of fusion protein obtained were free from any contaminating proteins that could be visualized by Coomassie blue staining (Figure 1A).

We have shown previously that a rabbit reticulocyte lysate supplemented with mevalonic acid and canine pancreas microsomal membranes can fully process *in vitro* translated K-ras(B) protein (Hancock *et al.*, 1991a). This system also processes mutant K-ras proteins with CAIL and CCIL C-terminal motifs which are geranylgeranylated, AAX proteolysed and methylesterified (Hancock *et al.*, 1991b; Newman *et al.*, 1992). The microsomal membranes provide a source of the AAX endoprotease which cleaves off the AAX sequence prior to methylation of the C-terminal cysteine residue by a methyltransferase, which is also present on the membranes. Rando *et al.* (Ma and Rando, 1992; Ma *et al.*, 1992) have demonstrated that the AAX endoprotease present on canine microsomal membranes can cleave both geranylgeranylated and farnesylated CAAX peptides. Taken together these data indicate that a rabbit reticulocyte lysate supplemented with canine pancreatic microsomal membranes and mevalonic acid can be used to translate RhoA and Rac1 proteins which will then undergo normal post-translational processing.

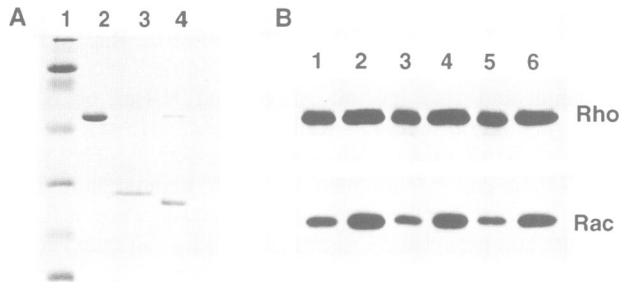

Fig. 1. (**A**) Purification of GST−GDI fusion protein. GDI−GST fusion protein was purified on glutathione agarose beads and cleaved with thrombin as described in Materials and methods. The procedure was analysed by SDS−PAGE and the gel stained with Coomassie blue. Lane 1, mol. wt markers: 14, 21, 30, 46, 69, 97 and 200 kDa; lane 2, 5 μg GST−GDI fusion protein, Predicted mol. wt = 53 kDa; lane 3, GDI released following thrombin cleavage of the fusion protein, mol. wt = 27 kDa; lane 4, analysis of the glutathione agarose beads following thrombin cleavage. GST (mol. wt 26 kDa) remains attached as does a small amount of uncleaved GDI−GST fusion protein. (**B**) *In vitro* processing of RhoA and Rac1 proteins. RhoA and Rac1 proteins were translated *in vitro* in [^{35}S]methionine labelled reticulocyte lysates and and 2 μl of the lysate partitioned in Triton X-114. The aqueous phases (lanes 1, 3 and 5) and detergent enriched phases (lanes 2, 4 and 6) were TCA precipitated, resolved on SDS gels and fluorographed. Lanes 1 and 2, RhoA (upper panel) and Rac1 (lower panel) translated in the absence of microsomal membranes. These proteins are geranylgeranylated but not AAX proteolysed or methylated. Lanes 3 and 4, RhoA and Rac1 proteins translated with microsomal membranes plus 10 μM farnesylthioacetic acid. These proteins are geranylgeranylated and AAX proteolysed but not methylated. Lanes 5 and 6, RhoA and Rac1 proteins translated with microsomal membranes. These proteins are fully processed. The figure illustrates that prenylation alone causes the protein to partition into the detergent enriched phase of Triton X-114 and that the proportion of detergent partitioning (i.e. partially or fully processed) protein is unaffected by AAX proteolysis and/or methylation. Quantification of the fluorogram shows that 60% translated RhoA and 73% translated Rac1 are geranylgeranylated in these reactions.

Prenylation and -AAX proteolysis are required for the binding of RhoA and Rac1 to GDI

We have shown previously that all three modifications of the CAAX motif are required for efficient membrane binding of K-ras(B) (Hancock *et al.*, 1991a). Similarly post-translational modification of Rho proteins has been shown to be essential for their localization and biological activity (Adamson *et al.*, 1992b). It was of interest therefore to determine which modifications, of geranylgeranylation, AAX proteolysis and methylesterification, contributed to the binding of RhoA and Rac1 to RhoGDI. Wild type RhoA (Gly14) and Rac1 (Gly12) mRNA were translated in rabbit reticulocyte lysates supplemented as described and labelled with [^{35}S]methionine to obtain fully processed proteins. To generate RhoA and Rac1 proteins which were partially processed, mRNA was translated without pancreatic microsomal membranes, or with these membranes in the presence of 10 μM farnesylthioacetic acid (FTA) which is a potent *in vitro* inhibitor of the methyltranferase (K_i = 0.1 μM) (Tan *et al.*, 1991). The proportion of processed RhoA and Rac1 protein present in the lysates was determined by partioning an aliquot of the reaction through Triton X-114. Figure 1B shows that 60−70% of RhoA and Rac1 is prenylated following translation and that AAX proteolysis and methylation do not increase the detergent partitioning fraction of either protein. This is consistent with previous observations on the *in vitro* processing of Ras proteins (Hancock *et al.*, 1991a).

Aliquots of these reticulocyte lysates were incubated with GDPβS to exchange the nucleotide present on the translated RhoA or Rac1 protein and then with GDI−GST fusion protein immobilized on glutathione agarose. Control incubations were carried out with GST immobilized on the same beads to determine non-specific binding. Figure 2 shows that geranylgeranylated, non-proteolysed RhoA and Rac1 bound to RhoGDI but to a much lesser extent than the fully processed proteins. However, the geranylgeranylated and proteolysed proteins bound efficiently to RhoGDI whether or not they were methylated. Indeed the data suggest that the non-methylated RhoA and Rac1 proteins may show some degree of enhanced binding to RhoGDI. RhoA with the mutation Cys190→Ser is blocked for all CAAX processing (Adamson *et al.*, 1992a). This mutant showed no binding to RhoGDI (Figure 3B), demonstrating that geranylgeranylation is absolutely required for the interaction in agreement with earlier reports that RhoGDI does not act on proteins lacking post-translational modification.

GDI binds the GTP and the GDP bound forms of RhoA and Rac

To determine whether RhoA and Rac1 could form complexes with RhoGDI in the GTP-bound form, aliquots of

reticulocyte lysate containing fully processed proteins were incubated with GDPβS or GTPγS to exchange the nucleotide on the translated protein. Lysates were incubated with GDI−GST fusion protein or GST protein immobilized on glutathione agarose beads. Figure 3A shows that RhoA and Rac in both the GDP and GTP bound forms bound to the GDI−GST fusion protein much more extensively than to the control GST protein. To semiquantify the binding efficiency an aliquot of each lysate was partitioned through Triton X-114 so that the amount of RhoA and Rac1 binding to the GDI−GST fusion protein could be expressed as a proportion of the processed protein present in the incubation. The detergent partioning fractions were precipitated with TCA and resolved on an SDS−PAGE gel alongside the RhoA and Rac1 captured on the beads. The fluorograms were scanned with an ImageQuant densitometer. Figure 3B shows that, after allowing for non-specific binding, 75% of RhoA-GDP and 52% of RhoA-GTP binds to RhoGDI whilst 65% of Rac-GDP and 88% of Rac-GTP bound to RhoGDI in the conditions of this assay. Thus both proteins bind to RhoGDI in the GDP and GTP bound states.

Mutations or ADP-ribosylation in the N-terminal effector domain do not block RhoGDI binding

We next investigated whether activating mutations of RhoA and Rac1, or inactivating mutations of Rac1 influenced binding to RhoGDI. Figure 3B shows that proteins with activating mutations, RhoA (Val14) and Rac1 (Val12) proteins have similar binding profiles to the wild type proteins when prebound with GDP or GTP. Two mutations in the N-terminal effector domain of Rac1 have been shown to produce biologically inactive proteins (C.Johnston and A.Hall, unpublished data). However, these two mutant proteins, Rac1 (Ala35) and Rac1 (Lys40) also bound RhoGDI to a similar extent as the wild type protein.

The C3 ADP-ribosyltransferase from *Clostridium botulinum* inactivates RhoA by ADP-ribosylation at Asn41 in the N-terminal effector domain (Aktories *et al.*, 1989; Ridley and Hall, 1992). We investigated whether ribosylation influenced the interaction of RhoGDI with RhoA. RhoA mRNA was translated in reticulocyte lysates containing microsomal membranes and labelled with [^{35}S]methionine to detect the translated protein. The lysate was then incubated with C3 transferase and [^{32}P]NAD$^+$. Approximately 50%

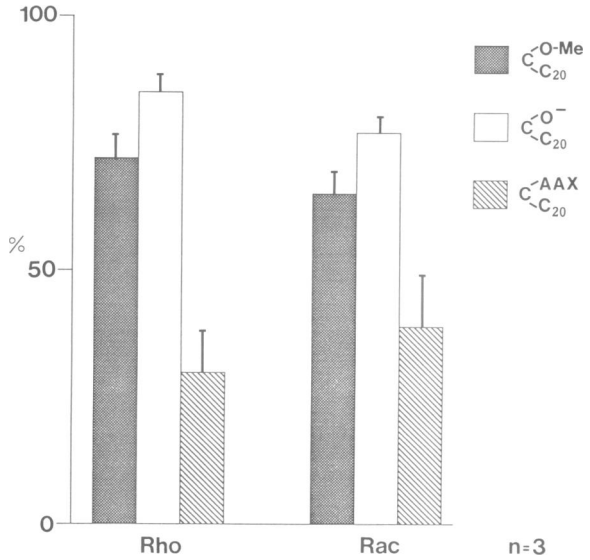

Fig. 2. *In vitro* binding of partially processed Rho and Rac proteins to RhoGDI. RhoA and Rac1 proteins were translated in [^{35}S]methionine labelled reticulocyte lysates supplemented with mevalonic acid to give geranylgeranylated, non-AAX proteolysed proteins. Microsomal membranes were used as a source of AAX protease and methyltransferase. 10 μM farnesylthioacetic acid was used to inhibit the methyltransferase on the microsomal membranes to give geranylgeranylated, AAX proteolysed but non-methylated proteins. The proteins were then prebound to GDP and incubated with RhoGDI−GST immobilized on glutathione agarose beads. After washing, the protein bound to the beads was visualized by SDS−PAGE. The amount of non-specific binding to the agarose beads was determined by carrying out control incubations with GST immobilized on glutathione agarose beads. 5−10% of the processed RhoA and Rac1 bound non-specifically to the GST beads and, as described in the legend to Figure 3, these counts were subtracted prior to tabulation. % = RhoA or Rac1 protein bound specifically to GDI; ×100%, total detergent partitioning RhoA or Rac1 in the incubation.

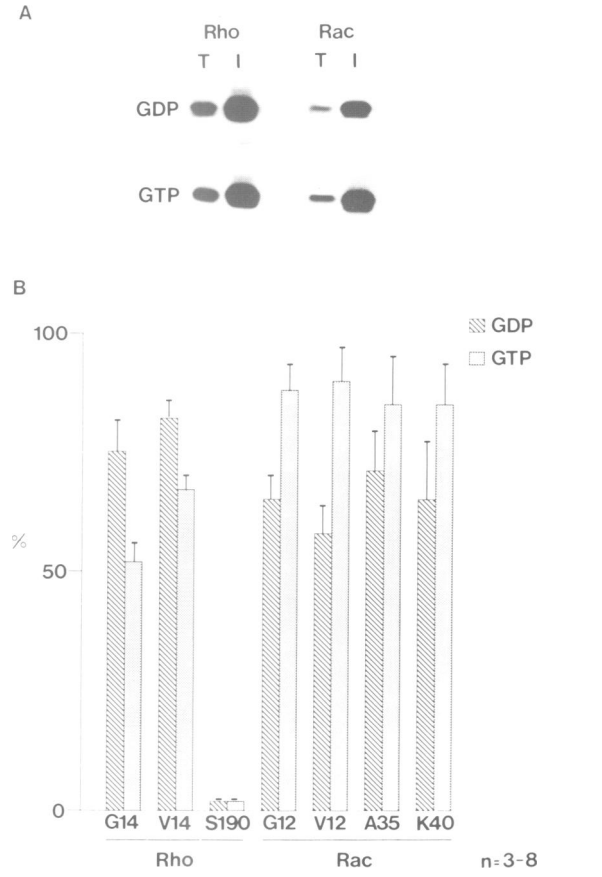

Fig. 3. (**A**) *In vitro* binding of RhoA and Rac1 proteins to RhoGDI. Wild type RhoA and Rac1 proteins translated in reticulocyte lysates supplemented with mevalonic acid and microsomal membranes and labelled with [^{35}S]methionine were prebound to GDP or GTP and then incubated with GST (T) or RhoGDI−GST (I) immobilized on glutathione agarose beads. After washing, the protein bound to the beads was visualized by SDS−PAGE. (**B**) Quantification of *in vitro* binding of RhoA and Rac1 mutant proteins to RhoGDI. Assays were performed as described in (A) and quantified either by counting radioactivity in gel slices or by densitometry of autoradiograms. The amount of processed protein available to bind to the fusion proteins (= 100%) was determined by partitioning an aliquot of the translation through 1% Triton X-114, TCA precipitation and SDS−PAGE. Counts bound to the GST beads (T in Figure 3A) were deemed to be non-specific and were subtracted from the counts on the GDI−GST beads (I in Figure 3A) before tabulation. As in Figure 2 some 5−10% of the detergent partitioning, prenylated proteins bound non-specifically to the GST beads. No non-specific binding was seen with the exclusively aqueous partitioning S190 mutant RhoA protein.

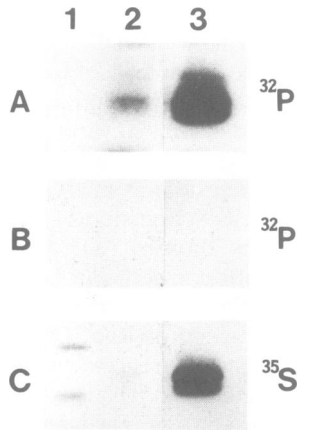

Fig. 4. Effect of ribosylation on binding of RhoA to RhoGDI. A reticulocyte lysate containing fully processed, ^{35}S-labelled RhoA was ribosylated with [^{32}P]NAD$^+$ using C3 transferase and prebound to GDPβS. The lysate was then incubated with GST immobilized on glutathione agarose beads for 15 min. The supernatant was removed and incubated with GDI–GST beads respectively for a further 15 min. The beads were then washed, boiled in Laemmli buffer and the proteins captured on the beads resolved by SDS–PAGE. The gel was fluorographed. Lane 1, mol. wt markers: 21 and 30 kDa; lane 2, proteins binding to the GST beads; lane 3, proteins binding to GDI–GST beads. (**A**) Gel screened with aluminium foil and exposed overnight at −70°C to detect ^{32}P label. (**B**) Re-exposure of the gel under the same conditions 2 months later showing decay of the ^{32}P label. (**C**) Re-exposure of the gel overnight following removal of the aluminium screens after decay of the ^{32}P label to reveal the ^{35}S label in the translated protein.

of RhoA is ribosylated under these conditions (Aktories *et al.*, 1989). The translated protein was prebound to GDPβS and incubated with GST or GDI–GST proteins immobilized on glutathione agarose beads. After washing, the beads were boiled in sample buffer and the proteins resolved by SDS–PAGE. The gel was screened with aluminium foil and exposed overnight to detect ^{32}P label (Figure 4A). The ^{32}P was then left to decay for 2 months until no signal was detected through the screen (Figure 4B). The screen was removed and the gel re-exposed to detect ^{35}S label (Figure 4C). The experiment shows that ribosylation of RhoA has no effect on its ability to bind to RhoGDI.

GDI blocks the action of RhoGAP and BCR on Rac1

Having shown that the GTP bound form of Rac1 binds to GDI we wished to investigate whether the binding of GDI, in addition to preventing nucleotide exchange, also prevented GAP stimulated GTP hydrolysis. To obtain larger amounts of processed protein, Rac1 protein with an N-terminal Myc epitope (Adamson *et al.*, 1992a) was transiently expressed in COS cells. Cell lysates were made in 1% Triton X-114 and the detergent partitioning (processed) and aqueous partitioning (unprocessed) proteins were immunoprecipitated separately with an anti-Myc monoclonal antibody and captured on protein G–Sepharose. After extensive washing the immunoprecipitated Rac1 proteins were bound to GTPγ^{32}P and split into incubations with or without recombinant RhoGDI. After 10 min, recombinant RhoGAP was added and the liberation of ^{32}P from the sepharose beads followed by scintillation counting the reaction supernatant. Control reactions comprised parallel incubations without RhoGAP. Figure 5A shows that the addition of RhoGAP to unprocessed and processed Rac1, immobilized on Sepharose beads, resulted in a significant release of ^{32}P into the supernatant compared with control incubations to which no RhoGAP was added. Preincubation with GDI blocked this release of ^{32}P but only in the sample containing post-translationally processed Rac1. RhoGDI had no effect on the GAP stimulated release of ^{32}P from unprocessed Rac1 and RhoGDI in the absence of RhoGAP did not itself stimulate any release of ^{32}P over background (Figure 5A). Identical results were obtained when this experiment was carried out with BCR protein in place of RhoGAP (Figure 5B).

Discussion

A feature common to the majority of Ras-related proteins is C-terminal prenylation. Ras proteins are farnesylated within a C-terminal CAAX motif which is then proteolysed and methylated and Ras-related proteins are geranylgeranylated within one of three alternative motifs (Hancock and Marshall, 1993). The post-translationally modified CAAX motif of Ras then acts in concert with an adjacent palmitoylation site or polybasic domain to target plasma membrane localization (Hancock *et al.*, 1990). Similarly, C-terminal prenylation is required for the binding of Ras-related proteins to membranes although the additional protein sequences which target these proteins to specific membranes have yet to be identified. The Rho subfamily of Ras-related proteins, Rho, Rac and CDC42 are involved in regulating the organization of the actin cytoskeleton (Ridley and Hall, 1992; Ridley *et al.*, 1992). Precisely how is not known, although most models call for reversible membrane interactions in order to allow cycling of the protein (Bourne *et al.*, 1991; Hall, 1990, 1992b). Geranylgeranylation, however, can bind proteins very avidly to cellular membranes to the extent that they are resistent to extraction with 1 M salt (Hancock *et al.*, 1991b). The demonstration that proteins such as RhoGDI and smgGDS can solubilize Rho-related proteins from membranes provides an elegant solution to the problem of how such an avid membrane association might be reversed without delipidating the C-terminus. Moreover since these same proteins can regulate nucleotide exchange it has been proposed that they also function as activators (smgGDS) or as inhibitors (RhoGDI) of Ras-related proteins (Fujioka *et al.*, 1992; Mizuno *et al.*, 1992). The role of RhoGDI in regulating Rac function is, however, unclear since a Rac–RhoGDI complex has been shown to be an active component of the NADPH oxidase activation pathway in neutrophils (Abo *et al.*, 1991).

We have shown previously that efficient binding of K-ras(B) to plasma membrane requires all three modifications of the C-terminal CAAX motif: farnesylation, AAX proteolysis and methylesterification of the C-terminal cysteine (Hancock *et al.*, 1991a). In contrast we have shown here that a significant proportion of geranylgeranylated, non-AAX proteolysed RhoA and Rac1 can bind to RhoGDI. The extent of this binding increased 2- to 3-fold following AAX proteolysis and decreased slightly following methylation. We have speculated previously that methylation of K-ras(B) increases plasma membrane binding because the C-terminal negative charge causes repulsion from negatively charged phospholipid headgroups. This may not be relevant to the binding of a lipidated C-terminus if the binding site in the GDI protein is not negatively charged.

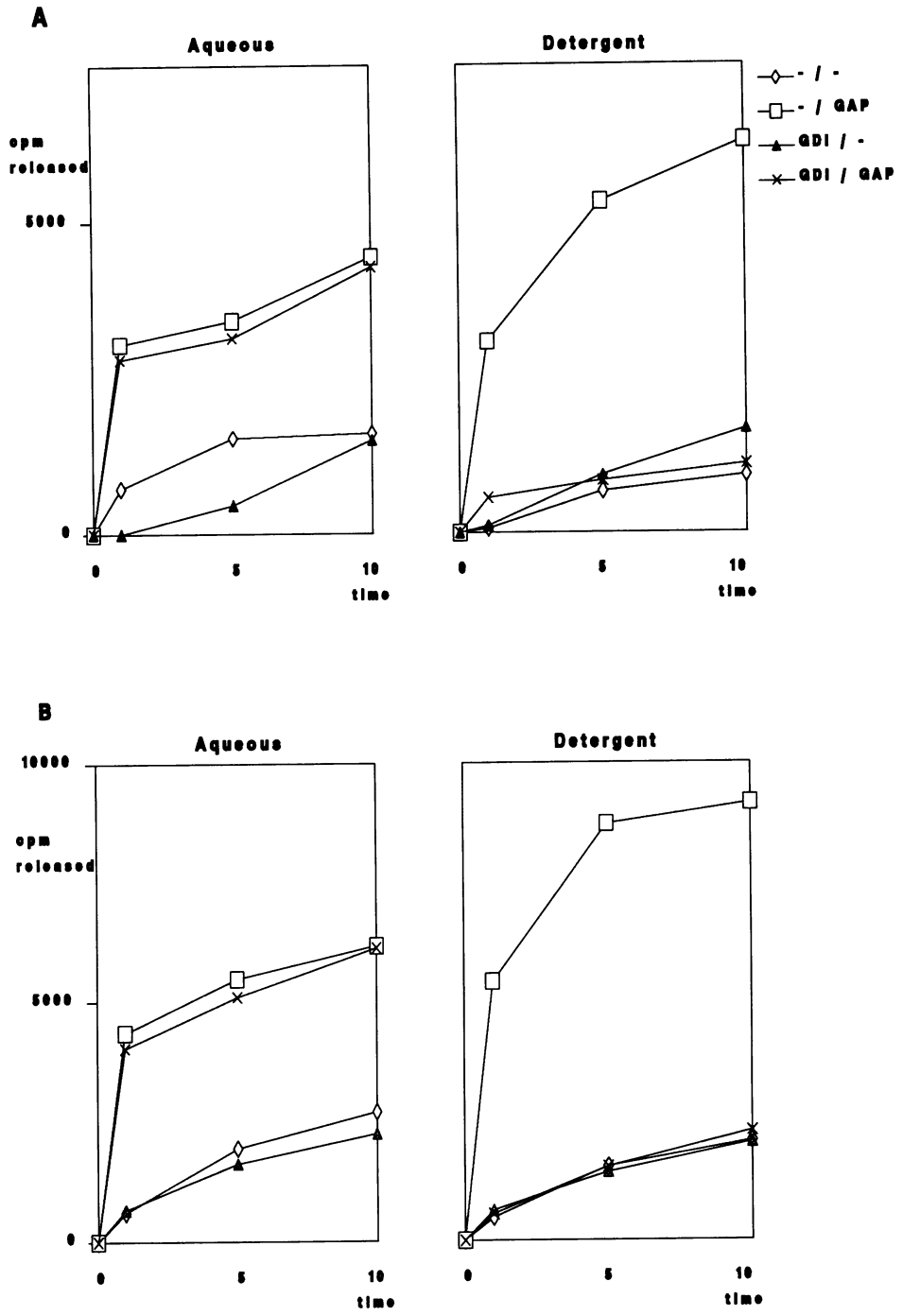

Fig. 5. RhoGDI inhibits GAP proteins. N-terminal Myc tagged Rac1 was expressed in COS cells and the cells lysed in 1% Triton X-114. The lysate was partitioned and the aqueous phase containing non-processed Rac1 protein and the detergent phase containing processed Rac1 protein immunoprecipitated spearately. The proteins immobilized on the beads were then loaded with GTPγ[^{32}P], washed extensively and split between four tubes. Recombinant RhoGDI was added to half the reactions and an equal ammount of BSA to the others. An aliquot of each of the supernatants was taken (t = 0) and recombinant RhoGAP or BSA added (**A**). Aliquots of the supernatants were taken at time intervals for scintillation counting from which the counts released from the beads after t = 0 were calculated. Panel **B** shows the same experiment repeated with recombinant Bcr in place of RhoGAP. At the end of the experiments quantitative Western blotting showed that 400 ng non-processed Rac1 and 600 ng processed Rac1 were present in the immunoprecipitations.

We have shown here that RhoA and Rac1 can form stable complexes with RhoGDI in the GDP and GTP bound states despite earlier reports to the contrary. In fact under the conditions of our *in vitro* assay RacGTP bound more extensively to RhoGDI than did RacGDP. Given that a Rac–RhoGDI complex has been purified from neutrophils that can activate NADPH oxidase, it seemed likely that the Rac protein in this complex was in the active GTP conformation. Our data show that this is indeed possible. RhoGDI itself, however, is not absolutely essential for activation of NADPH oxidase *in vitro* since recombinant Rac1 alone is active (Abo *et al.*, 1991). These data suggest

that RhoGDI serves some other function when complexed to Rac1 and we have now shown that GTP hydolysis stimulated by RhoGAP and BCR is blocked by prebinding RhoGDI to RacGTP. These experiments do not indicate the mechanism by which this GAP inhibition is achieved although the simplest model would be for the GDI protein to sterically hinder GAP binding. However, recent work by Hart *et al.* (1992) showed that a GDI for CDC42 inhibited both the GAP stimulated GTPase activity and the intrinsic GTPase activity of this Rho-related protein. GDI may, therefore, directly interfere with GTP hydrolysis on CDC42 and this result strongly suggests that RhoGDI operates in a similar way on Rac1. We did not observe an inhibition of intrinsic Rac1 GTPase activity by RhoGDI in this study but cannot exclude this possibility since the assay used was not sufficiently sensitive to detect such an effect reliably. Whatever the actual mechanism of action our data suggest that RhoGDI may have a novel function and act as a chaperone-like protein to transport activated Rac1 through the cytosol to its site(s) of action at a plasma membrane target. It is not clear at what point RhoGDI dissociates from Rac1 though it would seem likely that the binding of Rac1 to its target should trigger GDI release. Once dissociated from RhoGDI, RacGTP would then be accessible to GAP activity which would stimulate GTP hydrolysis returning Rac1 to the inactive GDP ground state.

Materials and methods

Cloning of RhoGDI

Human RhoGDI was cloned by PCR using normal human lymphocyte cDNA as template. The cDNA was prepared as described previously (Porfiri *et al.*, 1993). RhoGDI was obtained as a single PCR product using primers, designed from the published bovine sequence (Fukumoto *et al.*, 1990): 5'CGGGATCCATGGC(T/C)GA(G/A)CA(G/A)GA(G/A)CC(C/G)ACA-GCTGA(G/A)CA 5'GGAATTCT(C/T)A(G/A)TC(C/T)TTCCA(C/T)T-C(C/T)TTCTTGATGGT(G/T)AG. 5'*Bam*HI and 3'*Eco*RI restriction sites were included in the primers allowing the PCR product to be digested with these enzymes and cloned into into GEM-11Z(f−) (Promega). Three independent clones were fully sequenced. Outside of the primers the cDNA clones obtained the predicted seven amino acid changes from the bovine sequence. The cDNA was then subcloned into pGEX2T using the *Bam*HI/*Eco*RI sites in the polylinker of the plasmid.

Purification of GDI

GST−GDI fusion protein was purified essentially as described (Smith and Johnson, 1988). 10 ml bacterial cultures were grown to saturation overnight and then induced with 0.15 mM IPTG for 4 h. The bacteria were pelleted at 3000 *g*, washed in phosphate buffered saline (PBS) and resuspended in 2 ml PBS. The cells were gently sonicated on ice for 10 s twice and the cell debris removed by centrifugation at 10 000 *g* at 4°C. The supernatant was divided between two tubes and each 1 ml lysate incubated with 300 μl 50% suspension (in PBS) of washed glutathione agarose beads (Sigma) for 15 min at room temperature. The beads were washed six times with PBS and made up to a volume of 1 ml with PBS+0.02% azide after removal of the final wash. The beads were then stored at 4°C until required and used within 7 days. Control beads were obtained by transforming *E.coli* with GEX-2T plasmid and purifying the GST protein in the same manner.

500 ml cultures were used to prepare cleaved recombinant GDI for the GAP inhibition experiments. The same induction procedure was followed as outlined above. The cells were resuspended in 20 ml PBS and divided into five aliquots for sonication. The cleared lysate following the 10 000 *g* spin was incubated with 2 ml 50% suspension of glutathione agarose beads for 15 min and washed five times with 50 ml PBS, twice with 50 ml 50 mM Tris−Cl pH 7.5, 150 mM NaCl, once with 50 mM Tris−Cl pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$ and then resuspended in 1 ml of the final wash buffer. 100 μg human thrombin (Sigma) were added and the beads incubated at 25°C for 30 min. The supernatant and five 1 ml washes with 10 mM Tris−Cl pH 7.5, 50 mM NaCl were pooled and incubated at 4°C with 500 μl washed *p*-aminobenzamidine agarose beads (Sigma) to remove any free thrombin and then dialysed overnight against 10 mM Tris−Cl pH 7.5, 50 mM NaCl. The recombinant protein was quantified using the Bradford reaction, concentrated to 1 mg/ml, verified on an SDS gel and frozen in aliquots for storage at −70°C until required.

In vitro synthesis of RhoA and Rac1

RhoA and Rac1 cDNAs were cloned into pGEM vectors. All mutations were introduced by oligonucleotide mutagenesis and fully sequenced. An N-terminal Myc epitope (Adamson *et al.*, 1992b) was cloned on to the N-terminus of wild type RhoA and Rac1. Uncapped mRNA was transcribed *in vitro* from linearized plasmid templates using T7 or SP6 RNA polymerase. For *in vitro* translations 2 μg RNA were added to 50 μl of nuclease treated rabbit reticulocyte lysate (Promega) supplemented with 5 mM mevalonic acid as described previously (Hancock *et al.*, 1991a). 7.2 EQ of canine pancreatic microsomal membranes (Promega) were used as a source of AAX endopeptidase and methyltransferase in a 50 μl reaction (Hancock *et al.*, 1991a). Farnesylthioacetic acid (FTA) was provided by R.Rando (Harvard Medical School) and stored as a 500 μM stock in DMSO at −20°C; where required 1 μl was added to the translation reaction. Translations were otherwise performed according to the manufacturers instructions and labelled with [^{35}S]methionine (Amersham, SJ204; at 1.2 mCi/ml) in methionine-free amino acid mix. Unlabelled translations were carried out with a 1 mM mixture of all 20 amino acids.

Binding assays

To exchange the nucleotide present on the translated RhoA or Rac1 protein, 2.5 μl 60 mM GDPβS or GTPγS and 2.5 μl 30 mM EDTA were added to 25 μl labelled lysate. The mixture was incubated at 37°C for 10 min and the reaction stopped by placing it on ice and adding 30 μl ice cold TBM (20 mM Tris−Cl pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$). The concentration of Mg^{2+} in the Promega lysate is 500 μM against a final concentration of 2.5 mM EDTA in the exchange incubation. After adding TBM the final free Mg^{2+} concentration is >8 mM. The concentration of GTP and GDP in the lysate are <50 μM and hence the final concentration of GDPβS and GTPγS in the exchange reaction are >100 times greater at 5 mM. Non-hydrolysable GTP analogues were used since preliminary experiments demonstrated that the rabbit reticulocyte lysate contains RhoGAP activity.

200 μl of a 15% suspension in PBS of glutathione agarose beads with GST or GST−GDI fusion proteins bound (~15 μg) were centrifuged briefly and the supernatant replaced with 200 μl of TBAlb (20 mM Tris−Cl pH 7.5, 100 mM NaCl, 7.5 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, 100 μg/ml leupeptin, 10 μg/ml soy bean trypsin inhibitor, 10 μg/ml aprotinin) containing 1 mM GDP or GTP according to the nucleotide prebound to the translated protein. 17 μl of the lysate/TBM mix were added to the beads and the tubes mixed on a wheel at room temperature for 20 min. The beads were then collected by centrifugation at 16 000 *g* at 4°C for 1 min, washed with 1 ml ice-cold TBAlb, taken up in 1×Laemmli sample buffer and boiled.

For most experiments 17 μl of the lysate/TBM mix were added to aliquots of GST and GST−GDI beads and bindings to each carried out in parallel. 8.5 μl of the remaining lysate/TBM mix were added to 500 μl of 1% Triton X-114, incubated on ice for 10 min and then at 37°C for 2 min. The detergent phase containing hydrophobic, post-translationally processed protein was separated by a brief centrifugation at room temperature as described (Guterriez *et al.*, 1989). The detergent partitioning proteins were precipitated with 10% TCA, washed three times with acetone and taken up in Laemmli sample buffer as described elsewhere (Newman *et al.*, 1992).

The samples from the GST and GST−GDI bindings and the TCA precipitated proteins were resolved on a 15% SDS−polyacrylamide gel which was fixed and stained with Coomassie blue to verify the presence and integrity of the fusion proteins before being soaked in Enlightening (NEN), dried and autoradiographed. The extent of binding of RhoA and Rac1 was determined either by counting the radioactivity in gel slices as described previously (Hancock *et al.*, 1991a) or by using pre-flashed film and digitizing the fluorograms. With both methods the amount of post-translationally modified protein available in the incubation to bind to GDI was given by twice the signal from the TCA precipitated aliquot (= 100%). Non-specific binding to the GST/glutathione agarose beads was low (see figure legends) and these counts have been subtracted from the counts bound to the GDI−GST/glutathione agarose beads to arrive at the specific binding figures.

GAP assays

One 10 cm^2 dish of COS cells transiently expressing N-terminal Myc epitope-tagged wild type Rac1 was lysed in 1% Triton X-114 in TBS (10 mM Tris pH 7.5, 150 mM NaCl). The mixure was vortexed and incubated on ice for 10 min. The lysate was then partitioned and separated into aqueous and detergent phases as described previously (Gutierrez *et al.*, 1989). Each

phase was then washed by making it up to 1% Triton X-114 by the addition of 900 μl TBS to the detergent phase and 100 μl 10% Triton X-114 to the aqueous phase, incubating 10 min on ice and repartitioning. The wash ensures that there is mininimal contamination of the detergent phase with aqueous phase and vice versa. The Rac1 proteins were then immunoprecipitated from the washed aqueous and detergent phases with 50 μl 9E10 acitic fluid and 100 μl protein G−sepharose at 4°C for 2 h in the presence of a cocktail of protease inhibitors.

The beads were collected by centrifugation and washed five times with ice-cold 1% Triton X-114 in TBS and twice with ice-cold 20 mM Tris pH 7.5, 20 mM NaCl, 0.1 mM DTT, 2 mM EDTA. The beads were resuspended in 45 μl of the same buffer. 5 μl of GTP[γ-^{32}P] (Amersham, PB10244,) were added and the beads incubated at 30°C for 10 min with frequent mixing. They were then placed on ice and washed six times with ice-cold 10 mM $MgCl_2$, 20 mM Tris−Cl pH 7.5, 20 mM NaCl, 0.1 mM DTT, 2 mM Na_2PO_4, 0.1 mM GTP, 0.1 mg/ml BSA. During the final wash the beads were divided into four aliquots and resuspended in 45 μl of the same buffer. 5 μl of recombinant GDI 1 mg/ml were added to half the tubes and 5 μl of BSA 1 mg/ml to the other half. The beads were gently resuspended and after 10 min a 5 μl aliquot was removed as the time t = 0 count. 5 μl of recombinant RhoGAP or BCR 0.1 mg/ml was added to half the tubes and 5 μl BSA 0.1 mg/ml to the remainder. The tubes were incubated at 30°C with occasional resuspension and 5 μl aliquots of the supernatant removed at t = 1, t = 5 and t = 10 min for scintillation counting. Thus for each of the aqueous and detergent partitioning Rac1 fractions, incubations were performed with GDI ± RhoGAP and BSA ± RhoGAP. At the end of the experiment the amount of Rac protein present was determined by quantitative Western blotting against recombinant Rac using an anti-Rac serum.

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