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Identification of a GDI displacement factor that releases endosomal Rab GTPases from Rab–GDI

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Prenylated Rab GTPases occur in the cytosol in their GDP-bound conformations bound to a cytosolic protein termed GDP-dissociation inhibitor (GDI). Rab–GDI complexes represent a pool of active, recycling Rab proteins that can deliver Rabs to specific and distinct membrane-bound compartments. Rab delivery to cellular membranes involves release of GDI, and the membrane-associated Rab protein then exchanges its bound GDP for GTP. We report here the identification of a novel, membrane-associated protein factor that can release prenylated Rab proteins from GDI. This GDI-displacement factor (GDF) is not a guanine nucleotide exchange factor because it did not influence the intrinsic rates of nucleotide exchange by Rabs 5, 7 or 9. Rather, GDF caused the release of each of these endosomal Rabs from GDI, permitting them to exchange nucleotide at their intrinsic rates. GDF displayed the greatest catalytic rate enhancement on Rab9–GDI complexes. However, catalytic rate enhancement paralleled the potency of GDI in blocking nucleotide exchange: GDI was shown to be most potent in blocking nucleotide exchange by Rab9. The failure of GDF to act on Rab1–GDI complexes suggests that it may be specific for endosomal Rab proteins. This novel, membrane-associated activity may be part of the machinery used to localize Rabs to their correct intracellular compartments.

Keywords: Rab–GDI/Rab protein/Ras-like GTPase

Introduction

Rab proteins represent a family of at least 30 different Ras-like GTPases that function in transport vesicle docking with their targets (Novick and Brennwald, 1993; Zerial and Stenmark, 1993; Nuoffer and Balch, 1994). Almost every membrane-bound organelle in the secretory and endocytic pathways bears a distinct set of Rabs on its surface. Rabs are doubly geranylgeranylated at or near their C-termini, which is required for their membrane association. The specificity of Rab localization is provided by structural determinants unique to each family member (Chavrier *et al.*, 1991; Brennwald and Novick, 1993; Dunn *et al.*, 1993; Stenmark *et al.*, 1994).

At steady state, the bulk of a given Rab is membrane-associated, but a significant fraction is also detectable in the cytosol. All of the cytosolic pool represents Rabs bound to a small family of proteins termed Rab GDP-dissociation inhibitors (GDIs). After a vesicle fuses with its target, GDIs can retrieve Rabs from their fusion targets, in their GDP-bound conformations. GDIs also have the capacity to deliver Rab proteins to their specific membrane-bound compartments to enable them to function in vesicle docking processes (Pfeffer *et al.*, 1995).

Complexes of prenylated Rab5 or Rab9 proteins bound to GDI-α have been shown to be delivered to early or late endosomes respectively, in experiments using permeabilized cells (Ullrich *et al.*, 1994) or enriched membrane fractions (Soldati *et al.*, 1994). Membrane targeting was accompanied by release of GDI, followed by the exchange of Rab-bound GDP for GTP. Since membrane association seemed to precede the nucleotide exchange process, it was proposed that a novel activity that could displace GDI would catalyze the recruitment of Rab proteins onto specific organelle membranes (Pfeffer, 1994; Soldati *et al.*, 1994). In this model, nucleotide exchange would be catalyzed by a separate polypeptide that may or may not be physically associated with the GDI-displacement factor (GDF).

Evidence in support of the existence of a GDF comes from the work of Novick and colleagues, who showed that the Sec4p (yeast Rab) nucleotide exchange factor, Dss4p, could not use Sec4p–Gdip complexes as substrates (Moya *et al.*, 1993). Their findings indicated that an additional factor would be needed to act in concert with Dss4p, as part of the membrane recruitment process.

We looked for a nucleotide exchange-enhancing activity on late endosome membranes that could utilize Rab9–GDI complexes as substrates. We report here the >10 000-fold enrichment of an activity that can release intact, prenylated Rab9 from GDI. Once dissociated from GDI by this factor, the Rab exchanges nucleotide at its intrinsic rate. GDF acts on GDI complexes of other endosomally localized Rabs but not on complexes containing the endoplasmic reticulum (ER) and Golgi-associated Rab1b protein.

Results

Prenylated Rab proteins can be purified in CHAPS detergent from the membranes of insect cells infected with an appropriate, Rab-encoding baculovirus (Soldati *et al.*, 1995b). The purified Rabs can be retained in a soluble form upon removal of the detergent if delipidated serum albumin is added (Dirac-Svejstrup *et al.*, 1994). Presumably, the geranylgeranyl groups are bound by the serum albumin. We have shown that prenylated Rab9, in complex with bovine serum albumin (BSA), is very similar to unprenylated Rab9 in terms of its kinetic parameters

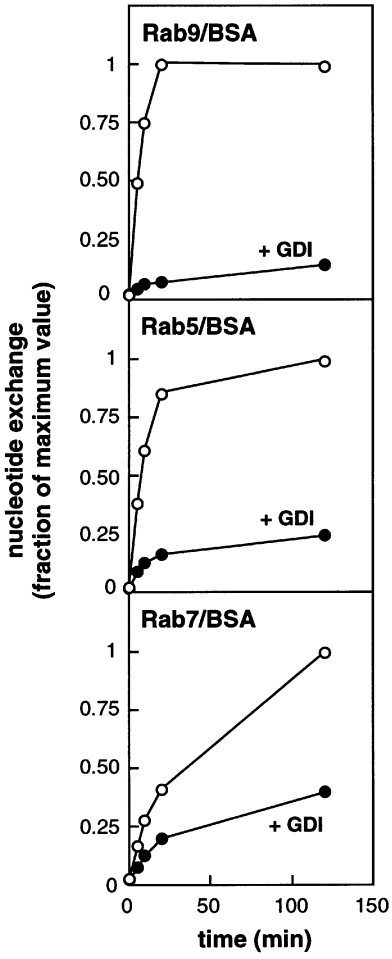

Fig. 1. GDI inhibits nucleotide exchange by different Rab proteins to different extents. Nucleotide exchange by purified, prenyl Rab9, Rab5 or Rab7 proteins, maintained in soluble form with delipidated BSA, was monitored in the presence or absence of equimolar GDI at 30°C. Maximum possible nucleotide exchange values for Rab9, Rab5 and Rab 7 were 17.9, 24 and 18.6 pmol GTPγS bound, respectively. Data were normalized to 1.0 to permit a direct comparison of the different Rab proteins, and represent the fraction of active nucleotide binding sites occupied for each Rab.

(Shapiro and Pfeffer, 1995) and ability to stimulate vesicular transport *in vitro* (Dirac-Svejstrup *et al.*, 1994).

As shown in Figure 1, purified, prenylated Rab9, complexed with BSA, was fully capable of exchanging bound, unlabeled GDP for [^{35}S]GTPγS (top panel, open symbols). Under the conditions of these experiments, essentially all of the Rab9 molecules added to the reactions exchanged nucleotide within 30 min at 30°C. A similar rate of exchange was observed for prenyl Rab5–BSA complexes (middle panel); Rab7 exchanged nucleotide more slowly, as we have reported previously (Shapiro *et al.*, 1993).

If an equimolar amount of GDI-α ('GDI') purified from bovine brain was added at time zero to such reactions, nucleotide exchange was inhibited significantly (Figure 1, solid symbols). The small, initial rise in Rab-associated nucleotide exchange observed in reactions containing GDI is likely to reflect the rate with which GDI associates with each Rab protein. GDI bound most rapidly to Rab9; it was much slower in binding to Rab7, and bound Rab5 at an intermediate rate. Moreover, GDI was most potent in inhibiting nucleotide exchange by Rab9. Comparison of the initial rates of nucleotide exchange for each Rab with the rate observed at apparent equilibrium in the presence of GDI indicated that GDI inhibited nucleotide exchange 95-, 72- or 13-fold for Rabs 9, 5 and 7, respectively (Table I).

Table I. Comparison of the ability of GDF and GDI to act on different Rabs

	Catalytic rate enhancement due to GDF	Fold decrease in GDP release due to GDI	Normalized GDF effect/GDI effect
Rab9	12 (1.0)	95 (1.0)	1.0
Rab5	6.5 (0.54)	72 (0.76)	0.7
Rab7	3.1 (0.26)	13 (0.14)	1.9

The catalytic rate enhancement for GDF on each Rab–GDI complex was determined by comparing the initial rates of nucleotide exchange of the complexes in the presence or absence of GDF. The ability of GDI to block GDP release from Rab proteins was determined by comparing the initial rates of nucleotide exchange by the prenylated Rab proteins, in complex with BSA, with that observed after subsequent addition of GDI and establishment of equilibrium. Shown in parentheses are the values normalized to that obtained for Rab9.

Identification of a GDF

Figure 2 outlines the assay we utilized in an attempt to detect a membrane-associated activity that would recognize a Rab–GDI complex and catalyze the release of GDI. We will refer to this as GDF or GDI-displacement factor activity. Rab proteins occur in the cytosol as complexes with GDI, which blocks their ability to release and exchange bound GDP. Once free from GDI, the Rab is then again able to exchange bound nucleotide, at least at the intrinsic rates shown in Figure 1. Since GDI binds tightly to Rab9 (Shapiro *et al.*, 1995), the equilibrium normally lies far in the direction of the stable Rab9–GDI complex. However, a GDF should facilitate the generation of free Rab9, which could then exchange bound GDP for [^{35}S]GTPγS.

We mixed stable Rab9–GDI complexes with protein fractions and looked for an increase in the rate of binding of [^{35}S]GTPγS. This method should have enabled us to detect a GDF, or an activity that acts on Rab–GDI complexes and stimulates that Rab's intrinsic rate of nucleotide exchange (a 'GEF'). A protein might also have the capacity to be both a GDF and a GEF.

Since GDI binds only prenyl Rabs, GDI release leads to Rab aggregation. Therefore, we carried out assays in the presence of detergent; a released Rab would become incorporated into a detergent micelle. Detergent conditions were established that do not destabilize prenyl Rab–GDI association (≤0.1% CHAPS in the final reaction mix). Moreover, since GDI shows a strong preference for Rabs bearing GDP, reactions were carried out in the presence of an excess of [^{35}S]GTPγS. Once released from GDI, a Rab will exchange bound GDP for GTPγS and then be incapable of rebinding GDI.

For these experiments, purified, prenylated Rab9, Rab5 and Rab7 in CHAPS were mixed with an equimolar amount of brain GDI and the detergent was then removed by dialysis. The stable complexes (Figure 3) were isolated

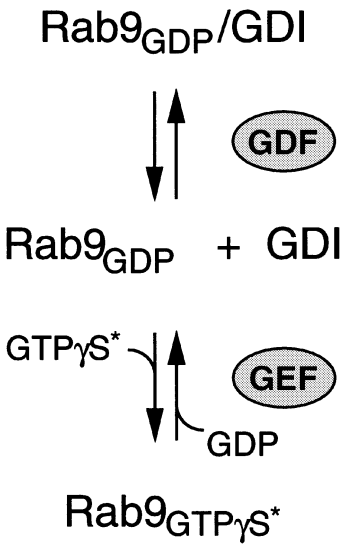

Fig. 2. Strategy for identifying a GDI displacement factor (GDF). Rab–GDI complexes are stable, but are in equilibrium with free Rab and GDI proteins. A GDF would act to catalyze the rate of this interconversion. Once released from GDI, a Rab could then exchange bound GDP for added [^{35}S]GTPγS. This could occur at the intrinsic rate of the Rab GTPase or at an enhanced rate, due to the action of a hypothetical GEF (guanine nucleotide exchange factor). Since GDI does not bind Rabs bearing GTP, the equilibrium can be shifted towards the formation of Rab9-GTP if reactions are carried out in excess GTPγS. This assay can detect either a GDF, a GEF that acts on Rab–GDI complexes, or both.

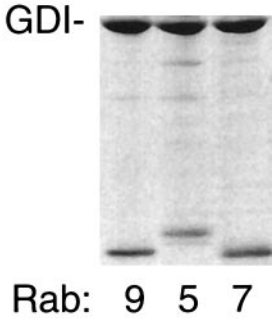

Fig. 3. Purified prenylated Rab–GDI complexes. An SDS–PAGE (12.5%) analysis is shown. GDI stains more darkly with Coomassie blue because it is twice the mass and also binds more dye than the prenyl Rab9 (see Soldati *et al.*, 1994, 1995a).

by gel filtration chromatography and eluted with an apparent mass of ~80 kDa.

Using this approach, we have identified a factor that can relieve the inhibition of nucleotide exchange by GDI. When Rab9–GDI complexes were incubated with an excess of GTPγS, very little exchange was observed (Figure 4A, open circles), as would be expected from the data shown in Figure 1. However, addition of significantly sub-stoichiometric amounts of our partially purified factor (⩽5 ng/μg Rab9) yielded a large increase in nucleotide exchange (Figure 4A, filled circles). The factor was partially purified from rat liver late endosome membranes that were pre-washed in 1 M KCl, solubilized in CHAPS detergent and fractionated by numerous chromatography steps; the specific activity of the factor was 2000-fold higher in the purified preparation compared with starting endosomes. Given the enrichment of endosomes, this is

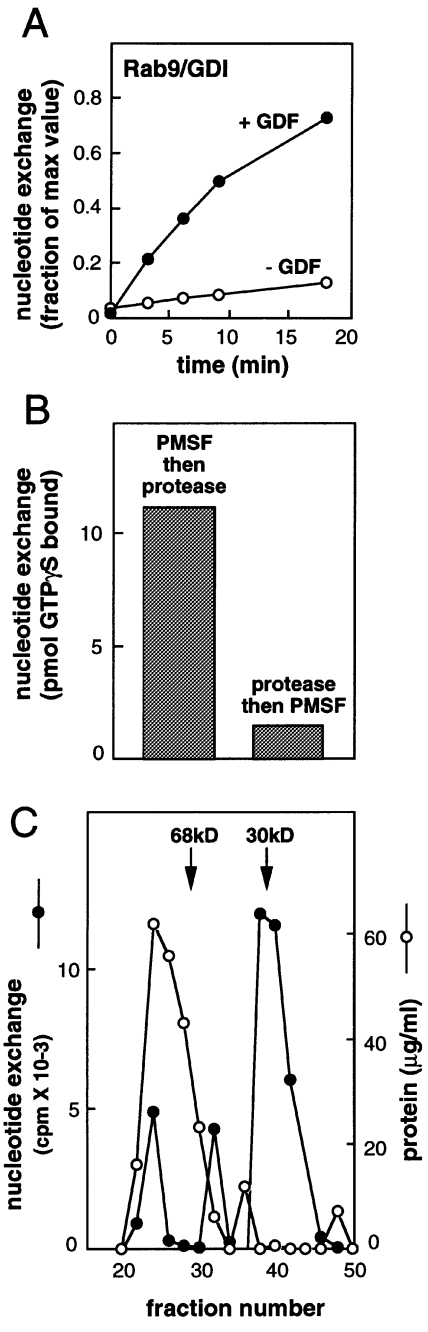

Fig. 4. (**A**) Identification of a proteinaceous factor that restores nucleotide exchange capacity to Rab–GDI complexes. Rab9–GDI complexes (20 pmol) were incubated in the presence or absence of 10 ng of factor for the indicated times at 37°C. The maximum possible nucleotide exchange value was determined by disrupting complexes in 5% CHAPS and allowing 2 h for subsequent nucleotide binding; it was 20 pmol GTP bound per 20 pmol Rab9. (**B**) The factor (10 ng) was incubated with 30 μl of proteinase K–Affigel (10 mg/ml) for 30 min at 37°C. PMSF was added to 1 mM either prior to bead addition or after the incubation. The ability of the factor to trigger nucleotide exchange by Rab9–GDI complexes was then measured in a reaction containing 20 pmol of Rab9 at 37°C for 20 min. (**C**) Superdex 75 gel filtration of GDF activity as described in Materials and methods. GDF activity (o) and protein (○) are shown. The mobilities of carbonic anhydrase (30 kDa) and serum albumin (68 kDa) are indicated.

likely to reflect at least a 10 000-fold purification relative to the post-nuclear supernatant. We have termed this factor GDF for GDI-Displacement Factor. As will be shown

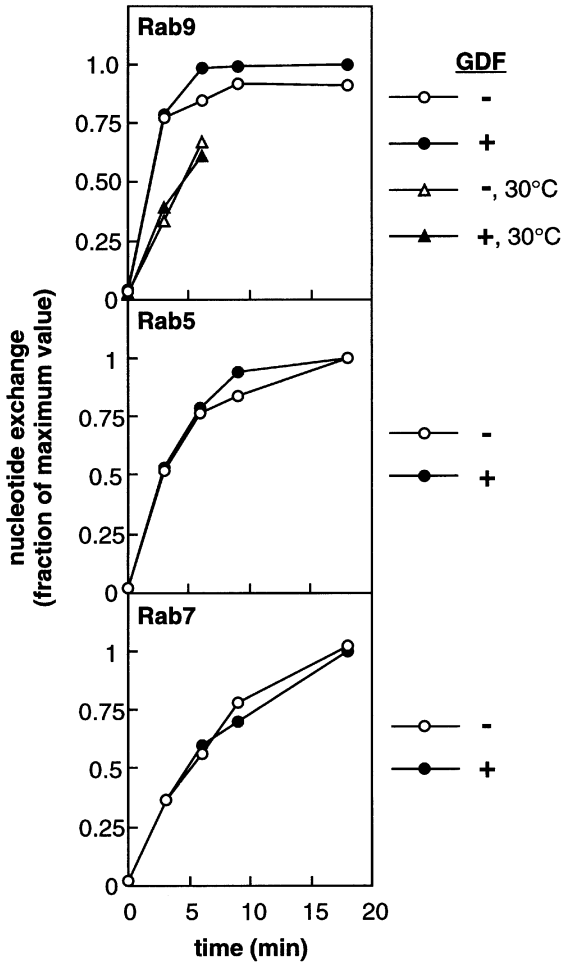

Fig. 5. Intrinsic nucleotide exchange rates are not influenced by the factor. Nucleotide exchange by prenyl Rab proteins (20 pmol) complexed with BSA was measured in the presence or absence of 10 ng factor at 37°C unless otherwise indicated. Maximum values were the same as indicated for Figure 1.

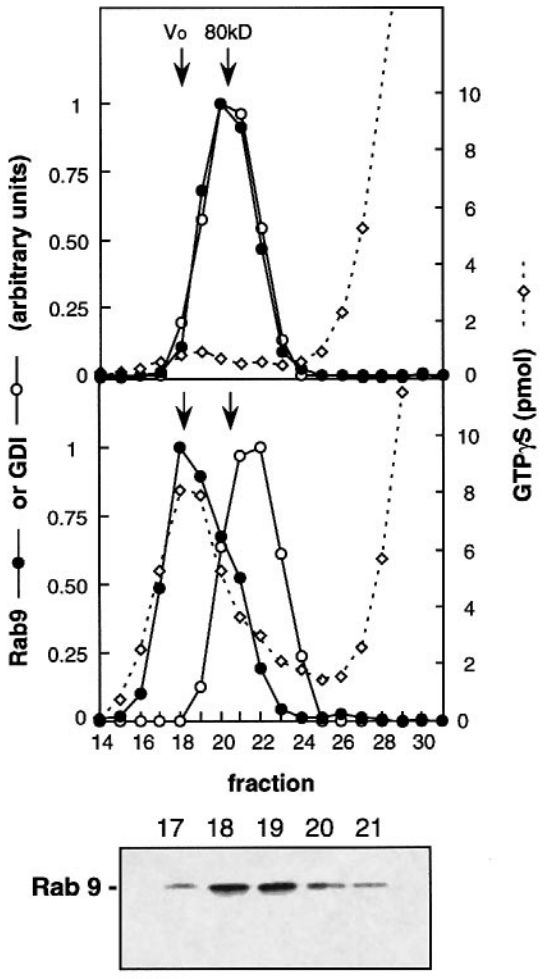

Fig. 6. Demonstration of a GDI-displacement factor. Prenyl Rab9–GDI complexes (40 pmol) were incubated with [^{35}S]GTPγS (560 nmol) in a volume of 100 µl for 30 min at 37°C. The reaction was then applied to a Sephacryl S100 column (2 ml column). Top panel, no factor added; middle panel, reaction carried out with 10 ng of factor. Fractions representing the void volume (V_o) and 80 kDa are indicated with arrows. The distributions of Rab9 and GDI were determined by quantitative immunoblotting and are presented in arbitrary units; [^{35}S]GTPγS distribution was determined by direct scintillation counting. Lower panel, immunoblot of Rab9 after GDF action. Data shown are from the experiment shown in the middle panel.

below, the factor could be shown to displace, physically, GDI from prenylated Rab9. Figure 4B shows the results of a proteinase K digestion; at least 85% of the activity was sensitive to protease treatment. Thus GDF is a protein. In addition, GDF activity chromatographed with an apparent mass of 30 kDa upon gel filtration (Figure 4C).

GDF is not a GEF

Several proteins have been identified that increase the intrinsic rate of nucleotide exchange by Rab GTPases (Burton *et al.*, 1993; Moya *et al.*, 1993; Horiuchi *et al.*, 1995): these are the so-called 'guanine nucleotide exchange factors' or GEFs. To determine if GDF had the ability to influence nucleotide exchange by Rabs, we examined nucleotide exchange of prenylated Rab–BSA complexes in the absence of GDI, with or without GDF. As shown in Figure 5, GDF (filled symbols) did not influence significantly the rate of nucleotide exchange by Rab9, either at 37 or 30°C. GDF also failed to act as a GEF for Rab5 or Rab7. These data suggest that GDF displaces Rabs from GDI, permitting them to exchange nucleotide at their intrinsic rates under the conditions of our assays. These data indicate further that GDI dissociation is rate limiting in assays of GDF activity.

GDF dissociates Rabs from GDI

The data presented thus far are consistent with a factor that can dissociate GDI from Rabs. If so, the reaction products should be resolvable by gel filtration chromatography. Rab–GDI complexes chromatograph with an apparent mass of ~80 kDa when fractionated on Sephacryl S100 columns (cf. Soldati *et al.*, 1993). If GDF dissociates the complexes, the GDI should now chromatograph as a ~55 kDa monomer and the free, prenylated Rab9 should elute with an apparent mass of ⩾100 kDa (Dirac-Svejstrup *et al.*, 1994). The large mass of free Rab9 is due to the formation of a small aggregate of the prenylated form since the column is run in the absence of detergent.

Figure 6 shows the results of chromatography of Rab9–GDI complexes, incubated either in buffer (top panel) or with catalytic amounts of GDF (middle panel). Reactions were carried out in the presence of excess [^{35}S]GTPγS. In the absence of GDF, Rab9–GDI complexes chromato-

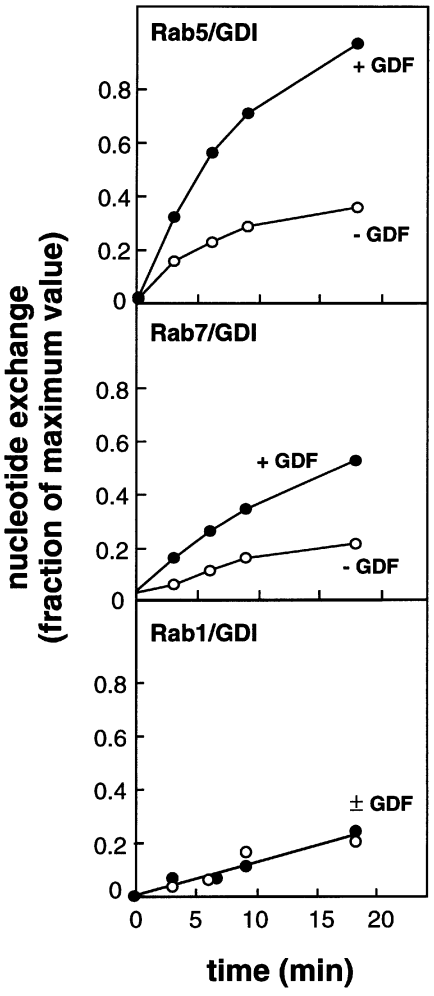

Fig. 7. Crude GDF can act on Rab5–GDI and Rab7–GDI complexes but not Rab1b–GDI complexes. Rab–GDI complexes (20 pmol) were incubated in the presence or absence of 10 ng of crude GDF for the indicated times at 37°C. Data are normalized to permit direct comparison; the maximum values were determined as in Figure 4. A gel of the Rab1–GDI complexes employed here is shown in Soldati *et al.* (1995a).

graphed with an apparent mass of ~80 kDa and, as expected, essentially no GTPγS was bound to those complexes (top panel). However, addition of ~10 ng of the GDF preparation to a reaction containing 1 μg of Rab9 bound to ~2 μg of GDI led to an almost complete shift in the distribution of Rab9 and GDI proteins. Rab9 was now capable of binding GTPγS and was well resolved from the bulk of GDI. Consistent with Rab9's ability to bind nucleotide, the immunoblot analyses used in these experiments documented that Rab9 (lower panel) and GDI (not shown) were fully intact at the end of the reaction. In summary, these experiments demonstrate, directly, the ability of GDF to catalyze the dissociation of Rab9–GDI complexes under the assay conditions described here.

Is GDF specific for Rab9?

GDF showed an apparent 12-fold catalytic rate enhancement using Rab9–GDI complexes as substrates (Figure 4, Table I). The ability of GDF to act on other Rabs was tested using complexes of prenylated Rab5 or Rab7 bound to GDI. Figure 7 shows that GDF also increased the

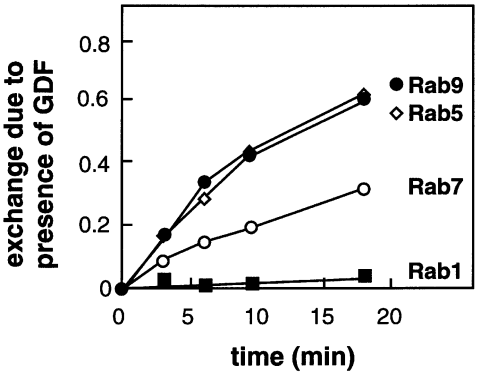

Fig. 8. Nucleotide exchange due to GDF action. The amounts of exchange measured for GDI complexes with Rab9, Rab5, Rab7 or Rab1b (from Figures 4 and 7) in the absence of GDF were subtracted from that seen in the presence of GDF and are plotted as a function of maximal exchange possible to permit a direct comparison.

apparent nucleotide exchange of Rab5–GDI and Rab7–GDI complexes. The increase in nucleotide exchange seen in the absence of GDF (Figure 7, open circles) reflects a slight instability of Rab5–GDI and Rab7–GDI complexes, compared with Rab9–GDI complexes (cf. Figure 4). When GDI falls off, nucleotide exchange occurs at the intrinsic rate of these Rab proteins. Despite this slight complex instability, GDF enhanced the observed exchange, presumably by displacing GDI, as was shown for Rab9 (Figure 6).

In contrast to the endosome-specific Rabs 5, 7 and 9, GDF failed to influence nucleotide exchange of Rab1–GDI complexes (Figure 7, bottom panel). The inability of GDF to act on Rab1–GDI complexes was not because these complexes are more stable; as shown in Figure 7, the complexes showed a spontaneous dissociation rate similar to that observed for Rabs 5 and 7. Moreover, Balch and colleagues (Schalk *et al.*, 1996) have estimated that the K_D for Rab1–GDI complexes is ~20-fold weaker than the values we have reported for Rab9–GDI complexes (Shapiro and Pfeffer, 1995).

Figure 8 presents the amount of exchange detected for each Rab–GDI complex in the presence of GDF, after subtraction of that seen in the absence of GDF. The data show that once released from GDI, the Rabs exchange nucleotide at their intrinsic rates; as mentioned earlier, Rab7 is known to exchange nucleotide at about half the rate of Rab9 (Shapiro *et al.*, 1993), and Rab5 exchange rates are comparable with those of Rab9 (Simon *et al.*, 1996).

GDF yielded rate enhancements of 6.5- and 3.1-fold, for Rab5–GDI and Rab7–GDI complexes, respectively, and 12-fold for Rab9–GDI complexes (Table I). At first glance, these data suggested that GDF showed a preference for Rab9. However, the relative catalytic action of GDF paralleled the strength of the GDI interaction with a given Rab. GDI bound most tightly to Rab9, and less tightly to Rab5 and Rab7 (Figures 1 and 7, Table I). Thus, when GDI bound a Rab tightly, crude GDF showed the greatest rate enhancement (Table I). When one compares GDF activity with GDI inhibition for each Rab, GDF showed only a modest preference for Rab9 compared with Rab5 (30%), and a 1.9-fold apparent preference for Rab7 compared with Rab9 (Table I). Nevertheless, it failed to act

on Rab1 complexes, consistent with a preference for endosomal Rab proteins.

Discussion

Prenylated Rab proteins occur in the cytosol bound to GDI, which has the capacity to deliver active Rabs to their specific, cognate membrane compartments (Pfeffer *et al.*, 1995). We have described here the identification of a novel, membrane-associated factor that can release GDI from prenylated Rab proteins when added in catalytic quantities. This is the first report of a protein that can detach Rabs from GDI, that may function in the process by which Rabs are delivered to distinct membrane targets. Since our factor was partially purified from endosome-enriched membrane fractions, it is likely to play a role in the recruitment of Rabs onto endosomes.

Prenylated Rab proteins first associate with membranes in their GDP-bound conformations, and it is only after several minutes that they bind GTP (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). Thus, while *in vitro* Rab recruitment experiments have shown that nucleotide exchange accompanies Rab membrane association, they have also suggested that the initial organelle recognition event may be more complex than simple interaction with a nucleotide exchange factor.

We proposed the existence of organelle-specific GDFs that might recognize distinct, prenylated Rabs, bound to GDI, and recruit the Rabs onto specific membranes (Pfeffer, 1994; Soldati *et al.*, 1994). Such factors could act catalytically: they might release GDI into the cytoplasm, enabling a prenylated Rab to associate spontaneously with the immediately adjacent membrane bilayer. A nucleotide exchanger would act on the membrane-associated Rab, which could then bind to some saturable, downstream component and function in vesicle targeting.

Evidence in support of this model has come from the work of Novick and co-workers, who identified a membrane-associated nucleotide exchanger for the yeast Sec4p Rab. This protein, termed Dss4p, stimulates the intrinsic exchange rate of Sec4p, but can also act on the yeast Ypt1p Rab, *in vitro* (Moya *et al.*, 1993). A related, mammalian protein, termed Mss4, shows similar GEF activity for a subset of Rabs (Burton *et al.*, 1993). It is important to note that Dss4p cannot act on complexes of Sec4p bound to yeast GDI. This suggests that another factor functions upstream of Dss4p to generate a substrate for this GEF. Since Dss4p showed only ~2-fold preference for Sec4p over Ypt1p, such an upstream factor would also be expected to confer some degree of specificity to the Rab targeting process.

Zerial and co-workers have reported an activity on bovine brain clathrin-coated vesicles that triggers nucleotide exchange when GDI–Rab5 complexes are utilized as substrates (Horiuchi *et al.*, 1995). Although GEF activity was very clearly demonstrated, these workers did not evaluate whether their protein had GDF activity. However, unlike the clathrin coated vesicle-associated factor or Dss4p and Mss4, our factor fails to influence the intrinsic rates of nucleotide exchange of three different prenylated Rab proteins, including Rab5 and Rab7. Thus, as isolated, GDF does not possess GEF activity and, thus far, shows no indication of interaction with free, prenylated Rab proteins.

If the GDF activity described here acts upstream of a Dss4p-like nucleotide exchanger, what can we say about its apparent, predicted specificity? Under the conditions of our *in vitro* experiments, GDF acted on endosomal Rabs 5, 7 and 9 but not on the ER/Golgi Rab, Rab1b. GDF displayed the greatest catalytic rate enhancement on complexes of prenylated Rab9 bound to GDI-α. At first glance, one could conclude that GDF acted preferentially on Rab 9. However, it is essential to note that Rab5 and Rab7 proteins formed weaker complexes with GDI-α after *in vitro* reconstitution. This is despite the fact that we do not employ His-tagged GDI or epitope-tagged Rab5 or Rab7 proteins, which we have found weaken GDI–Rab protein interactions. Thus, at least under the conditions of these experiments, when the endosomal Rab–GDI complex was tight, GDF dissociated it; when the complex was weaker, a lower rate of enhancement was detected, but the complex was dissociated, none the less. In all cases, GDI displacement was rate limiting. Thus, as isolated to date, GDF does not appear to show a significant level of discrimination between these endosomal Rabs, but it fails to act on the ER/Golgi Rab, Rab1b.

We have shown previously that Rab9 and Rab7 are each recruited onto endosome membranes with approximate apparent K_m values of 9 and 22 nM, respectively (Soldati *et al.*, 1995a). However, while control Rab9–GDI-α complexes inhibited the initial rate of myc-tagged Rab9 recruitment with an apparent K_i of ≈9 nM, Rab7 complexes inhibited this process much less effectively (apparent K_i ≈112 nM). Similarly, complexes of the ER-localized Rab1b protein were even less potent than Rab7 complexes (apparent K_i ≈405 nM). These experiments distinguished biochemically the recruitment of different Rab proteins onto a single class of organelle, and suggested that the membrane recruitment 'entry site' was Rab specific. As isolated thus far, GDF is able to discriminate between the endosomal Rabs and Rab1b, consistent with our previous competition experiments (Soldati *et al.*, 1995a).

The GDF described here acts on Rab proteins localized to early and late endosomes. It is possible that endosomal Rabs are recruited onto a common endosome and achieve their final steady-state distributions by an endosomal membrane transfer process. Alternatively, early versus late endosome specificity in Rab recruitment may be imparted by the concerted action of GDF with a GEF, rather than by either component alone. Moreover, it is still possible that our not yet homogeneous GDF preparation may contain distinct early and late endosomal GDFs with similar biochemical properties.

Finally, the overall specificity of Rab recruitment may also be influenced by the rate with which Rabs are converted to their GTP-bound forms. If a Rab-GDP is mislocalized, GDI can remove it and attempt to deliver it to another membrane compartment. In this regard, Rab5 and Rab7 hydrolyze GTP with $t_{1/2}$s of 6 min and 2.1 h, respectively (Simon *et al.*, 1996). If Rab5 were mislocalized and acted upon by a low specificity nucleotide exchanger, Rab5-GTP would convert spontaneously to Rab5-GDP, a substrate for GDI removal. In contrast, mislocalization of the slower Rabs 9 and 7 GTPases would be more difficult to correct. Such differences in kinetic properties of Rabs could have significant consequences in terms of the Rab cycle; in this manner, GDI may play

a significant role as a correction factor during Rab-targeting events.

In summary, we have reported here the existence of a novel enzyme activity that can displace endosomal Rab proteins from GDI. Our next challenges include identification of the proteins that interact with Rabs to promote nucleotide exchange and downstream vesicle docking events.

Materials and methods

GDI was purified from bovine brain according to Sasaki *et al.* (1990). Protein was measured according to Bradford (1976) or by use of bicinchoninic acid (Pierce Chemical Co.) using BSA as standard. Lipid was measured according to Lanzetta *et al.* (1979). SDS–PAGE was carried out according to Laemmli (1970). Human Rab5 cDNA and Rab1b-encoding baculovirus were the gifts of Dr M.Wessling-Resnick (Harvard University) and Bill Balch (Scripps Research Institute), respectively. Anti-Rab5 antibody was obtained from Santa Cruz Biotechnology.

Preparation of prenyl Rab–GDI and prenyl Rab–BSA complexes

Prenyl Rab proteins were obtained after expression in insect cells using standard baculovirus techniques as described (Soldati *et al.*, 1995b). Prenyl Rab9–GDI, Rab7–GDI, Rab5–GDI and Rab1–GDI complexes were reconstituted with 95% efficiency by dialyzing purified, prenylated Rab with an equimolar amount of purified bovine brain GDI, followed by Sephacryl S100 gel filtration chromatography (Soldati *et al.*, 1995b). Fractions containing Rab–GDI complexes were pooled, and aliquots were quickly frozen and stored at –80°C with 1.0 mg/ml BSA as carrier. Prenylated Rabs were purified to >90% homogeneity in a three step purification protocol (Soldati *et al.*, 1995b) starting from membranes of baculovirus-infected insect cells solubilized in 2% CHAPS, followed by chromatography in 1% CHAPS on Sephacryl S100 and MonoQ.

Rab–BSA complexes were assembled by dialyzing pure prenylated Rabs in the presence of a 10-fold molar excess of delipidated BSA (Sigma). After ultracentrifugation to remove some residual, non-BSA-complexed (and probably aggregated) Rab, the mass of the resulting complexes was determined by gel filtration chromatography to be ~100 kDa. Quantitation of Coomassie blue-stained gels indicated that Rab–BSA complexes were comprised of 2 or 3 mol of prenyl Rab/mol of BSA. Aliquots were stored at –80°C.

GDF purification

Late endosome membranes were obtained from frozen rat livers (Harlan Bio Products) as described by Tabas and Kornfeld (1979). These were stripped in 1 M KCl by adding 1 volume of 3 M KCl and 1 volume of ddH_2O, followed by incubation at room temperature for 15 min. Stripped membranes were pelleted by ultracentrifugation at 35 000 r.p.m. for 2 h at 4°C in a Ti45 rotor in a Beckman ultracentrifuge. The pellet was washed in 20 mM Tris pH 8.0, 10 mM NaCl, 8 mM $MgCl_2$, 2 mM EDTA, to reduce the KCl content. Membranes were solubilized by douncing in a 10-fold weight excess of CHAPS (Sigma) over protein in 20 mM Tris pH 8.5, 2 mM EDTA and 8 mM $MgCl_2$ (QFF buffer); usually ~80 ml of a 10% CHAPS buffer was used. The extract was centrifuged at 3700 r.p.m. in a GPR Beckman centrifuge for 15 min and then fractionated by anion-exchange chromatography (QFF Sepharose, Pharmacia, 1 ml resin/10 mg protein) in 20 mM Tris pH 8.5, 2 mM EDTA and 1% CHAPS. The column was eluted with a gradient of 10–600 mM NaCl. Active fractions were dialyzed to a conductivity equivalent of 10 mM NaCl against QFF buffer, concentrated on a small QFF Sepharose column by step elution in QFF buffer, 600 mM NaCl, containing a 10-fold weight excess of CHAPS over protein. The eluate was loaded on an S100 or Superdex 75 Pharmacia gel filtration column, which was run in 64 mM Tris pH 8.0, 100 mM NaCl, 8 mM $MgCl_2$, 2 mM EDTA and 1% CHAPS. The peak fraction was dialyzed into 10 volumes of 20 mM Tris pH 9.0, 1% CHAPS and then concentrated on a 1 ml MonoQ Pharmacia FPLC anion-exchange column pre-equilibrated in QFF buffer + 10 mM NaCl; the eluate was made 1% octylglucoside (Boehringer) and dialyzed into 75 mM Tris-OAc, pH 9.3, 1% octylglucoside and fractionated by chromatofocusing on a Pharmacia MonoP column. A pH gradient of 9.0 to 6.0 was obtained using 75 mM Tris, pH 9.3, 1% octylglucoside as start buffer and Polybuffer96, pH 6.0, 1% octylglucoside as elution buffer. The active fractions were concentrated using MonoQ as before. This fraction was used for all experiments described herein and represents a 2000-fold increase in specific activity over the initial endosome-enriched fractions. The fraction had a specific activity of 833 pmol GTPγS/μg of protein, as determined in a 20 min assay.

Nucleotide exchange assay for purification and characterization of GDF

Nucleotide exchange was assayed in 100 μl mixtures which contained 0.2 μM (20 pmol) Rab complex (BSA or GDI), and 5.5 μM [^{35}S]GTPγS (0.5 μCi). Reactions were carried out in 20 mM HEPES, pH 7.2, 90 mM KCl, 1.2 mM MgOAc, 100 mM $(NH_4)_2SO_4$, 1× protease inhibitor cocktail (Goda and Pfeffer, 1988) and 0.1% CHAPS. GDF was added and reactions proceeded for the times and at the temperatures indicated. Reactions were stopped on ice by addition of 500 μl of ice-cold 64 mM HEPES pH 8.0, 2 mM EDTA, 8 mM $MgCl_2$, 100 mM NaCl. [^{35}S]GTPγS bound to Rab proteins was monitored by filter binding followed by scintillation counting (Shapiro *et al.*, 1993). Background due to the addition of 0.1% CHAPS-containing buffer to GDI complexes was subtracted (<10% of maximum counts bound).

Protease sensitivity of GDF

Proteinase K (2 mg) was coupled to 200 μl of Affigel 10 (BioRad; 10 mg proteinase K/ml resin) in 0.1 M HEPES, pH 7.4 at 4°C. The resin was blocked in 0.1 M Tris, pH 8.0. For protease digestion, 30 μl of Affigel beads was incubated with 10 ng of GDF for 20 min at 37°C in 20 mM Tris, pH 8.0; the beads were removed and 1 mM phenylmethylsulfonyl fluoride (PMSF, from a 100× stock in absolute EtOH) was added. In control experiments, PMSF was added prior to proteinase K.

GDI displacement

Nucleotide exchange was carried out under standard conditions. Rab9 (40 pmol = 1 μg) in complex with GDI was incubated with either 10 ng of GDF or QFF buffer, in the presence of [^{35}S]GTPγS (10 μM) in a volume of 100 μl for 30 min at 37°C. Reactions were loaded directly onto a 2 ml Pharmacia S100 gel filtration column run in 64 mM HEPES, pH 8.0, 8 mM $MgCl_2$, 2 mM EDTA with no detergent added; 33 fractions (60 μl) were collected. The content of [^{35}S]GTPγS in each fraction was monitored by scintillation counting of 5 μl of each fraction. Fractions 14–31 (30 μl) were analyzed by 12.5% SDS–PAGE and quantitative immunoblotting (Burnette, 1981; Soldati *et al.*, 1993).

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