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Characterization of a guanine nucleotide-releasing factor and a GTPase-activating protein that are specific for the *ras*-related protein $p25^{rab3A}$

(*ras*-related guanine nucleotide-binding protein)

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ABSTRACT **The *rab3A* gene product is a 25-kilodalton guanine nucleotide-binding protein that is expressed at high levels in neural tissue and has about 30% homology to the *ras* gene product. Recombinant Rab3A protein and $p25^{rab3A}$ purified from bovine brain membranes have been used as substrates to look for factors that regulate its biochemical activity. A factor in rat brain cytosol exists that accelerates, by ≈10-fold, the release and subsequent rebinding of guanine nucleotides to both native and recombinant $p25^{rab3A}$. We have partially purified this activity, termed Rab3A-GRF, and a GTPase-activating protein (Rab3A-GAP) reported previously. The two activities copurified through a variety of procedures but were separated by Mono Q anion-exchange chromatography, indicating that the activities arise from distinct polypeptides. Both factors were thermolabile, sensitive to trypsin, and specific for Rab3A, exhibiting little or no activity toward c-Ha-Ras or Rab2 proteins. By gel filtration chromatography and sucrose density ultracentrifugation, both Rab3A-GRF and Rab3A-GAP have Stokes radii of 79 Å and sedimentation coefficients of 8.9 S. We calculate a molecular mass of 295,000 daltons and a frictional ratio of 1.80 for each factor.**

Guanine nucleotide-binding proteins (G proteins) function as molecular switches to control a wide variety of cellular processes (1–4). Hydrolysis of bound GTP switches these proteins between their active (GTP bound) and inactive (GDP bound) conformations. The hydrolysis rate and GDP dissociation rate provide a regulatable timing device that determines how long a G protein is "on." Factors that stimulate intrinsic GTPase activity have been discovered for several small (*ras*-related) GTPases (5–10). Whether or not these GTPase-activating proteins (GAPs) also represent the effector molecules, or "targets," for the small GTPases is not clear (11, 12). Other factors, called guanine nucleotide-releasing factor (GRF), *ras* guanine nucleotide exchange factor (rGEF), and GDP dissociation stimulator (GDS), have been described that accelerate release of GDP and subsequent rebinding of GTP to Ras and Rap1 proteins (13–15). These factors have been proposed to serve as "activators" of small GTPases.

$p25^{rab3A}$ (smg25A) is a small G protein of unknown function, with ≈30% sequence homology to $p21^{ras}$ (16). We (17) and others (18–21) have shown that $p25^{rab3A}$ is expressed at detectable levels only in exocrine tissues and is distributed approximately equally between cytosolic and particulate fractions, similar to the YPT1 and SEC4 proteins in yeast (22, 23). Here we report the use of recombinant Rab3A protein and anti-Rab3A antiserum to characterize a Rab3A-specific GRF and a previously described Rab3A-specific GAP (24).

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MATERIALS AND METHODS

Materials. [α-^{32}P]GTP (≈3000 Ci/mmol; 1 Ci = 37 GBq) was from DuPont/NEN. Protease inhibitors and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate were from Boehringer Mannheim. Protein A-Sepharose was from Sigma. Rat brains were purchased from Bioproducts for Science, Indianapolis. Other reagents were from standard vendors.

Cell Fractionation. Frozen rat brains were diced into small pieces, thawed, and immediately placed in ice-cold buffer A (2 ml/g of brain), containing 20 mM Mops (pH 7.1), 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and the following protease inhibitors: phenylmethylsulfonyl fluoride (10 μg/ml), leupeptin (10 μg/ml), aprotinin (10 μg/ml), and pepstatin A (1 μg/ml). The material was homogenized and centrifuged at 100,000 × *g* for 120 min; the supernatant was reserved as the cytosolic fraction. Membranes were prepared as described (24).

GRF Assays. Binding of [α-^{32}P]GDP to $p25^{rab3A}$ was assayed in buffer B (50 mM Hepes, pH 7.4/100 mM KCl/1 mM dithiothreitol/10 mM EDTA with insulin (1 mg/ml) and labeled nucleotide (0.5–1.0 μCi/μl). After 5 min at 30°C, $MgCl_2$ (15 mM final concentration) was added to stabilize the nucleotide–protein complexes. Nucleotide-protein complex (1–2 μl) was then added to reaction buffer (20 μl) containing 50 mM Tris·HCl (pH 8.0), excess nonradioactive GTP (2 mM), and 10 mM $MgCl_2$, in either buffer A or cytosol. Reaction mixtures were incubated at 30°C. Aliquots (2–5 μl) of the reaction mixture were removed at intervals and assayed for GRF activity by one of three methods. *Method 1, GDP release.* Aliquots of the reaction mixture were immunoprecipitated in 40 μl of an ice-cold solution containing protein A-Sepharose suspended in buffer C (25 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM EDTA, 1 mM dithiothreitol/260 mM sucrose) and precoupled to anti-Rab3A antibodies. The samples were rotated for 10 min at 4°C, washed thrice with 700 μl of ice-cold buffer C, and analyzed for loss of radioactivity by liquid scintillation counting. *Method 2, GDP release.* This was the same as method 1 except that the aliquots of reaction mixture were filter-bound as described (25). *Method 3, GDP binding.* This was the same as method 1 except that $p25^{rab3A}$ was bound to unlabeled GDP (200 μM) and added to reaction buffers containing 10 μCi of [α-^{32}P]GDP instead of 2 mM GDP. Aliquots were removed and analyzed as in method 1 for an increase in radioactivity. Cytosol was precleared with anti-Rab3A antibodies precoupled to protein A-Sepharose except where noted. Cytosolic filtrate was used instead of control buffer to ensure that the total GDP concentration was identical to that in cytosol. Cytosolic filtrate was prepared by centrifugation of cytosol in

Abbreviations: GAP, GTPase-activating protein; GRF, guanine nucleotide-releasing factor.

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Centricon 10 filter units (Amicon), which did not allow GRF to pass through the filters.

Purification Procedures. *Ammonium sulfate precipitation.* Cytosol prepared as described above was dialyzed for 4 hr at 4°C with stirring against a volume of 50% saturated ammonium sulfate in buffer A such that upon reaching equilibrium, the final concentration of ammonium sulfate in the cytosol was 35%. All Rab3A-GRF and Rab3A-GAP activity was recovered in the precipitate. The solution was centrifuged at 12,500 × *g* for 20 min. The supernatant was carefully decanted and the precipitate was redissolved in buffer D (50 mM Tris·HCl, pH 7.5/1 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol, dialyzed overnight against 2 liters of buffer D, and stored at −80°C until use. *Mono Q chromatography.* Material that had been fractionated by ammonium sulfate precipitation was filtered through a 0.22-μm filter and applied to a Mono Q HR5/5 column equilibrated in buffer D. The column was run at 0.25 ml/min and 0.5-ml fractions were collected. The column was washed with 5 ml of buffer D plus 50 mM NaCl, developed with a 10-ml linear NaCl gradient (50–550 mM), and further washed with 5 ml of buffer D plus 1 M NaCl. This procedure was repeated several times. The majority of GRF activity was eluted in fractions 24 and 25. The majority of GAP activity was eluted in fractions 26 and 27 (see Fig. 4). Use of shallower gradients increased the resolution of GRF from GAP only slightly. Active fractions were either pooled or kept separate before being concentrated in Centricon 30 filtration units and stored at −80°C until use. *Gel filtration chromatography.* Either crude cytosol or active fractions from the Mono Q column were concentrated in Centricon 30 filtration units and loaded onto a Superose 12 column equilibrated with buffer D plus 0.1 M NaCl. The column was run at 0.2 ml/min and 0.5-ml fractions were collected. *Sucrose density ultracentrifugation.* Either crude cytosol or active fractions from the Mono Q column (pooled or separate) were concentrated in Centricon 30 filtration units, loaded onto 5-ml linear sucrose gradients (8–24% or 13.5–32%) in buffer E (50 mM Pipes, pN 6.8/0.5 mM EDTA/40 mM NaCl), and centrifuged at 360,000 × *g* for 2 hr in a Beckman VT-80 rotor. Fractions (0.2 ml) were collected by vertical displacement with an ISCO fractionator. Method 2 was used to detect Rab3A-GRF activity in most procedures, and methods 1 and 3 were used for verification. GAP activity was assessed by using thin-layer chromatography to quantitate the rate of conversion of GTP to GDP bound to Rab3A, as described (24). Fractions from ion-exchange procedures were first dialyzed to remove salt before analysis. All procedures were carried out at 4°C.

Other Methods. Production of recombinant $p25^{rab3A}$ has been described (24). Characterization of anti-Rab3A antiserum will be described elsewhere (K. Linko-Stentz and I.G.M., unpublished work). Conversion of [α-^{32}P]GTP to [α-^{32}P]GDP was performed as described (26).

RESULTS

Recently, factors have been discovered that accelerate the release of guanine nucleotides from, and their subsequent rebinding to, the Ras and Rap small GTPases. These factors are known as GRF (13, 14) and GDS (15), respectively. However, no such factors have been described that regulate the Rab class of small G proteins. To determine whether such a factor exists that is specific for $p25^{rab3A}$, we incubated [α-^{32}P]GDP–$p25^{rab3A}$ with rat brain cytosol, or buffer A, and measured the off-rate of [α-^{32}P]GDP. Cytosol accelerated the off-rate of [α-^{32}P]GDP ≈10-fold (Fig. 1*A*) as measured by method 2. To be sure this was not an artifact of filter-binding, we verified the results by method 1 (data not shown; see *Materials and Methods*).

To show that the accelerated off-rate could be of physiological significance, and to rule out proteolysis of $p25^{rab3A}$, it was important to show that cytosol also accelerated exchange of guanine nucleotides *onto* $p25^{rab3A}$. As shown in Fig. 1*B*, cytosol accelerated nucleotide exchange onto $p25^{rab3A}$ to an

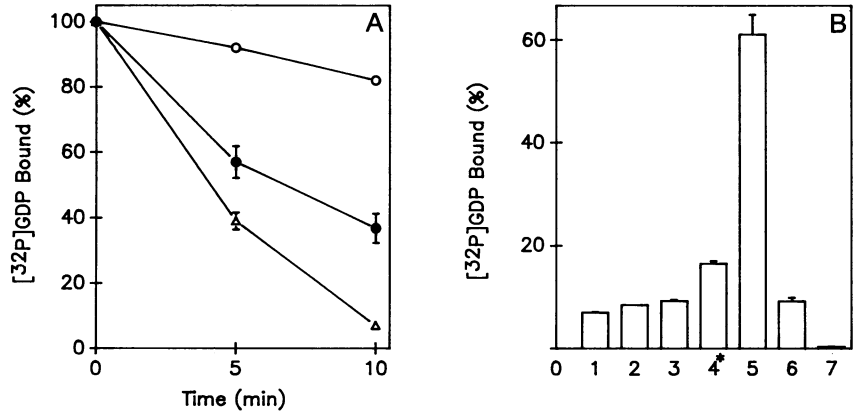

FIG. 1. Catalysis of guanine nucleotide exchange of Rab3A. (*A*) Cytosol catalyzes release of [α-^{32}P]GDP from Rab3A. Recombinant Rab3A protein was bound with [α-^{32}P]GDP as described in *Materials and Methods* and incubated in the presence of buffer (○), cytosol at 6 mg/ml (●), or cytosol at 16 mg/ml (△), each with 2 mM GDP and 10 mM $MgCl_2$. Aliquots were removed at the indicated time intervals and analyzed by filter binding. Each data point represents the mean ± SEM of three determinations. Results have been normalized to the [α-^{32}P]GDP bound at time 0 and are expressed as percent bound. (*B*) Cytosol catalyzes nucleotide exchange. Rab3A protein was bound with unlabeled GDP (200 μM) as described, added to 50 μl of either cytosol or cytosolic filtrate, each containing 10 μCi of [α-^{32}P]GDP and 10 mM $MgCl_2$, and incubated for 10 min at 30°C. $p25^{rab3A}$ was then immunoprecipitated, washed three times in buffer A plus 10 mM $MgCl_2$, and analyzed for [α-^{32}P]GDP bound. Cytosolic filtrate was used to ensure that the concentration of GDP was the same in control buffer as in cytosol. To assess background binding, equivalent GDP without $p25^{rab3A}$ was added to the incubation mixture instead of GDP·$p25^{rab3A}$. To demonstrate specificity, preimmune serum instead of immune serum was used for the immunoprecipitation. In all cases, except where noted by an asterisk, cytosol was first precleared of endogenous Rab3A protein with immune serum antibodies coupled to protein A-Sepharose. Maximum (defined as 100%) binding of [α-^{32}]GDP to exogenous Rab3A protein was assessed by adding an equivalent amount of GDP·$p25^{rab3A}$ to cytosolic filtrate containing 10 μCi of [α-^{32}P]GDP and 10 mM EDTA. Results shown were normalized to this value (150,000 dpm; data not shown) and represent the mean ± SEM of four determinations. The ingredients in the incubation mixtures were as follows. Bar 1, precleared cytosol, GDP alone, preimmune serum; bar 2, precleared cytosol, GDP·$p25^{rab3A}$ preimmune serum; bar 3, precleared cytosol, GDP alone, immune serum; bar 4, cytosol, GDP alone, immune serum; bar 5, precleared cytosol, GDP·$p25^{rab3A}$, immune serum; bar 6, cytosolic filtrate, GDP·$p25^{rab3A}$, immune serum; bar 7, cytosolic filtrate, GDP alone, immune serum.

extent comparable to the acceleration of nucleotide release. The enhanced binding seen in cytosol was not due to endogenous Rab3A protein; preclearing of cytosol with anti-Rab3A reduced binding to levels seen when preimmune serum was used (Fig. 1*B*, compare lanes 1–4). The exchange of [α-^{32}P]GDP onto p25^{rab3A} was completely blocked by the nonhydrolyzable GTP analogue guanosine 5'-[γ-thio]triphosphate (data not shown), indicating that the exchange factor could serve to activate p25^{rab3A} *in vivo* by facilitating exchange of GTP for GDP.

Others have been unable to detect GRF activity toward p25^{rab3A} (13, 27). However, in both cases, p25^{rab3A} purified from bovine brain membranes was used as the substrate, whereas we used recombinant Rab3A protein. Thus, it was important to demonstrate that rat brain cytosol could accelerate release of guanine nucleotides from endogenous as well as from recombinant Rab3A. Mammalian p25^{rab3A} is found distributed between cytosolic and membrane fractions (17). Rat brain cytosol, solubilized membranes, and purified recombinant p25^{rab3A} were immunoprecipitated with an antiserum to the *rab3A* gene product. The washed immunoprecipitates were loaded with [α-^{32}P]GDP and incubated with either control buffer or cytosol. The antibody did not alter the basal exchange rate of guanine nucleotide (data not shown). All three forms of p25^{rab3A} served as substrates for Rab3A-GRF activity (Fig. 2).

Superose 12 gel filtration of crude cytosol (Fig. 3) revealed that Rab3A-GRF activity comigrated with the Rab3A-GAP activity we reported previously (24). Virtually identical results were obtained when material was used that had been partially purified through ammonium sulfate precipitation and either Mono Q or Mono S column chromatography (data not shown). This result suggested to us that Rab3A-GAP and Rab3A-GRF either were distinct activities of a single polypeptide, or comprised separate polypeptides associated as part of a large complex, or were separate polypeptides with similar physical characteristics. To distinguish these possibilities we attempted to separate GRF from GAP by a variety of procedures. We were not able to resolve Rab3A-GRF from Rab3A-GAP by ammonium sulfate precipitation, DEAE or

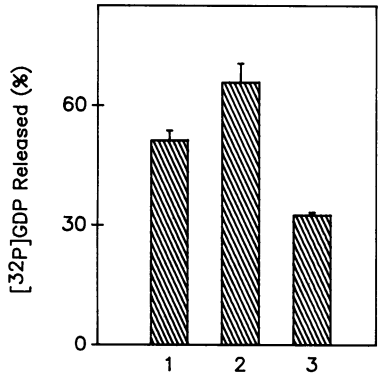

FIG. 2. Rab3A-GRF catalyzes release from recombinant and mammalian forms of Rab3A. Immunoprecipitates were prepared from cytosolic (bar 1) or membrane (bar 2) fractions of rat brain or from purified, recombinant p25^{rab3A} (bar 3) by using an antiserum against p25^{rab3A}. The pellets were washed twice in buffer A plus 1 M NaCl and then twice in buffer A. Immunocomplexes were bound to [α-^{32}P]GDP as described in *Materials and Methods* and washed three times in buffer A plus 10 mM $MgCl_2$. Immobilized [α-^{32}P]GDP complexes were then incubated with either buffer or cytosol, each containing 2 mM GDP and 10 mM $MgCl_2$, for 10 min at 30°C, washed three times, and assayed for radioactivity; 285,000, 240,000, and 300,000 dpm represent 100% bound for cytosolic, membrane-bound and recombinant Rab3A, respectively. Results are the mean ± SEM of three to five determinations. Results are expressed as [1 − (dpm bound$_{cytosol,t=10}$)/(dpm bound$_{buffer,t=10}$)] × 100%.

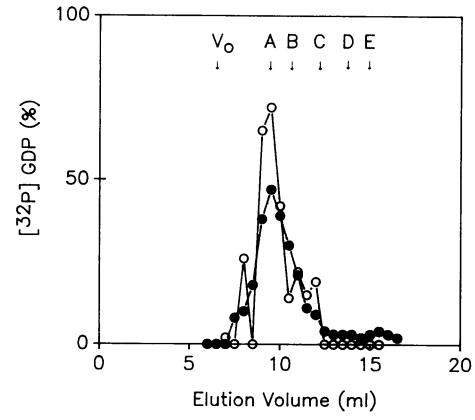

FIG. 3. Gel filtration on Superose 12. Crude cytosol was concentrated in a Centricon 10 (200 μl, 40 mg/ml), loaded onto a Superose 12 sizing column, and eluted with buffer D plus 0.1 M NaCl. Fractions (0.5 ml) were collected and assayed for Rab3A-GRF activity by method 1 (○) or for Rab3A-GAP activity (●) as described (25). The column was calibrated with A, apoferritin (440 kDa); B, β-amylase (200 kDa); C, bovine serum albumin (66 kDa); D, carbonic anhydrase (29 kDa); and E, cytochrome *c* (12 kDa). V_o, void volume.

Mono S chromatography, or sucrose density ultracentrifugation.

We were finally able to resolve Rab3A-GRF from Rab3A-GAP by Mono Q chromatography (Fig. 4), indicating that they are distinct entities. Nonetheless, the possibility remained that GRF and GAP could physically associate, gen-

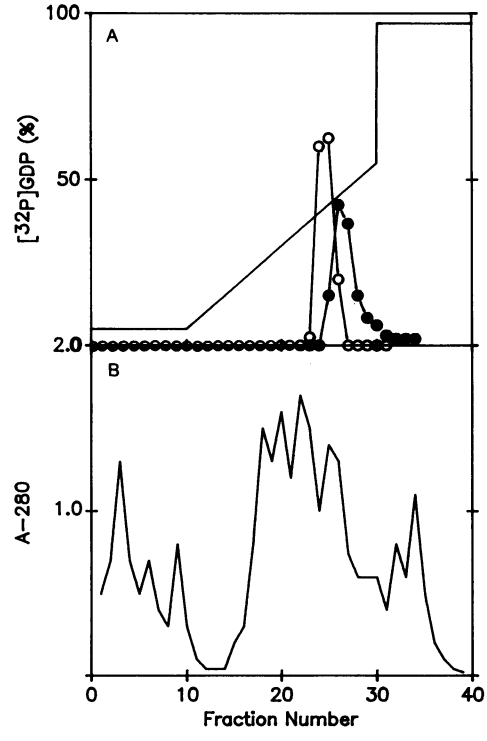

FIG. 4. Mono Q chromatography. Protein from the 0–35% ammonium sulfate fraction was dialyzed against 2 liters of buffer C for 24 hr with one buffer change and applied to a Mono Q HR5/5 column. The column was washed with 5 ml of buffer D plus 50 mM NaCl, followed by a 10-ml linear NaCl gradient (50–550 mM), and buffer D plus 1 M NaCl. (*Upper*) Fractions (0.5 ml) were collected and assayed for Rab3A-GRF activity by method 2 (○) or for Rab3A-GAP activity (●) as described (24). Percent recovery of activity was 54% for GRF and 47% for GAP. The elution profile shown was extremely reproducible and is representative of five runs. (*Lower*) Absorbance at 280 nM.

erating the high molecular weight species observed on gel filtration and sucrose density centrifugation. The sucrose density result was particularly suggestive, since both activities sedimented anomalously at 8.9 S (Fig. 5A). To settle this issue, we loaded either ammonium sulfate-purified material (data not shown) or fractions from the Mono Q column containing GRF activity (fraction 24) or GAP activity (fraction 27), either separately or together, onto sucrose gradients, and determined s values in each case. We estimated the cross-contamination of GRF and GAP to be ≤10%. The sedimentation coefficient for each activity was the same (Fig. 5B), independent of the purity of the sample (data not shown), the presence of the other activity, or the density of the gradient (note that different gradients were used in Fig. 5 A and B). Thus we conclude that Rab3A-GRF and Rab3A-GAP are entirely separate entities with similar physical characteristics. Using a value of 79 Å for the Stokes radius determined by gel filtration and a sedimentation coefficient of 8.9 S, and assuming a partial specific volume of 0.725 ml/g for each activity (a valid assumption since different density gradients did not affect the s value of either activity), we calculated a molecular mass of 295,000 daltons and a frictional ratio of 1.80 for Rab3A-GRF and Rab3A-GAP (28).

Further data on the characterization of Rab3A-GRF and Rab3A-GAP are presented in Table 1. Both activities are specific for Rab3A, moderately heat-labile, and sensitive to trypsin. We detected Rab3A-GAP activity in both the cytosolic and particulate fractions of rat brain (24) but detected Rab3A-GRF only in the cytosol.

DISCUSSION

At present, regulatory/effector proteins for the small GTPases are largely unknown (5–10, 13–15, 27, 29). We now report the identification of a cytosolic factor that stimulates the release of guanine nucleotides from $p25^{rab3A}$ as well as their subsequent rebinding. This factor, Rab3A-GRF, stimulates the off-rate of GDP by about 10-fold in a crude preparation (Fig. 1) and >20-fold in a partially purified state (data not shown), comparable to the effect of Ras-GRF upon $p21^{Ha\text{-}ras}$ (13) and much greater than the effect of Rap1-GDS on the Rap1 protein (15).

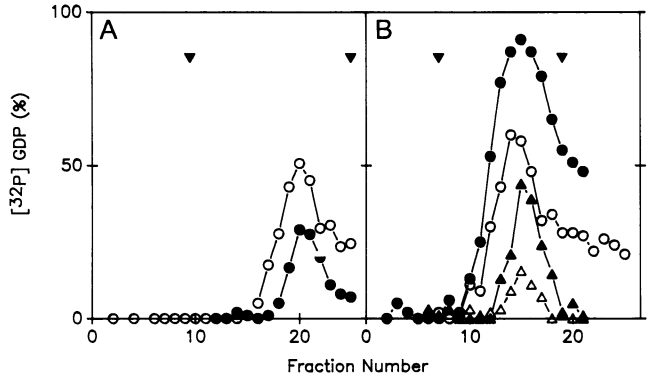

FIG. 5. Sucrose density ultracentrifugation. (A) Fractions from the Mono Q column containing both GRF and GAP activity were pooled, concentrated 10-fold in a Centricon 30 filtration unit, layered onto a 5-ml, 8–24% sucrose gradient in buffer E, and centrifuged in a vertical rotor (Beckman VT-80) for 2 hr at 70,000 rpm. Fractions (0.2 ml) were collected and assayed for GRF (○) and GAP (●). Standards were run together with the samples and are denoted by inverted triangles (ovalbumin, 3.6 S; catalase, 11.3 S). (B) As for A, except that the gradient was 13.5–32%, and fractions containing GRF and GAP were concentrated and loaded either separately (filled symbols) or together (open symbols). Circles, GRF; triangles, denote GAP. Percent recoveries of activity were 35% in each case and were independent of whether or not GAP and GRF were loaded separately or together.

Table 1. Characterization of Rab3A-GRF and Rab3A-GAP

Condition	% GRF activity	% GAP activity
Specificity		
Rab3A	100	100
c-Ha-Ras	12	0
Rab2	0	0
Distribution		
Cytosol (10 mg/ml)	100	100
Membranes (10 mg/ml)	0	106
Controls		
Buffer alone	0	0
+ cytosol (10 mg/ml)	100	100
100°C/10 min	0	0
60°C/30 min	0	0
37°C/60 min	51	31
Trypsin (1 mg/ml) for 20 min, then trypsin inhibitor (5 mg/ml)	6	0
Trypsin inhibitor only	95	100
+ purified GEF (20 ng)	0	–
+ recombinant Ras-GAP (0.5 μg)	–	0
+ NF1 fusion protein (1 μg)	–	0

About 200 ng of purified recombinant Rab3A, c-Ha-Ras, and Rab2 were bound to [α-^{32}P]GDP and used as substrates. Incubation time for GRF assays was 10 min except for the specificity assays, which were for 20 min. GAP assays were for 6 min. Results are the mean of two experiments. GRF data were calculated as [GRF activity measured under the stated condition]/[GRF activity in cytosol (10 mg/ml)] × 100%. GAP data were calculated as [GAP activity measured under the stated condition]/[GAP activity in cytosol (10 mg/ml)] × 100%. Active fractions from the Mono Q column (Fig. 4) were used to assess specificity. GEF is the exchange factor for eukaryotic initiation factor 2. The NF1 fusion protein is a portion of the neurofibromatosis 1 gene product fused to glutathione transferase, and possesses Ras-GAP activity.

We recently reported a Rab3A-specific GAP that accelerates the GTPase activity of Rab3A (24). In this paper we have shown that Rab3A-GRF and Rab3A-GAP are distinct, separate proteins that exhibit strikingly similar physical characteristics including chromatographic behavior, molecular mass, and shape. From the gel filtration and sucrose density experiments, it appears that both are highly asymmetric proteins (frictional ratio, 1.8) and >10-fold larger than Rab3A itself. Interestingly, other proteins associated with neural synapses are known to be highly asymmetric (30).

A third regulatory factor for $p25^{rab3A}$ has been described, GDI, which binds specifically to the GDP-occupied state of the protein and which at low Mg^{2+} concentrations inhibits GDP release (27, 31). Interestingly, rab3A-GDI appears to interact with the C-terminal region of endogenous $p25^{rab3A}$, but not with recombinant $p25^{rab3A}$, possibly through association with the posttranslationally modified C-terminal cysteine residues (32). In contrast, Rab3A-GRF appears to interact with both forms, as determined by immunoprecipitation assays (Fig. 2).

GDI will catalyze the dissociation of GDP·$p25^{rab3A}$ from membranes (27). Therefore, one can envision a cyclical process in which the cytosolic GDP·$p25^{rab3A}$·GDI complex interacts with GRF, triggering the exchange of GDP for GTP and the release of GDI. The GTP·$p25^{rab3A}$ would then interact with its target (vesicle?) membrane, possible by association with Rab3A-GAP, which could function as both downstream effector and negative regulator. Hydrolysis of bound GTP, catalyzed by GAP, would complete the cycle by converting the membrane-bound $p25^{rab3A}$ to a state in which it could once again interact with GDI. The efficiency of cycling could be controlled by the distribution of GAP between the cytosol and membrane.

The function of this putative cycle remains unknown. There is preliminary evidence that $p25^{rab3A}$ may be involved in stimulus-coupled secretion (33). However, we have been unable to detect any significant redistribution of $p25^{rab3A}$ following depolarization of synaptosomes (D. Bielinski, K. Linko-Stentz, I.G.M., and R. E. Fine, unpublished work). Further insight into the functions of Rab3A-GRF and Rab3A-GAP will require their purification to homogeneity.

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