Resolution of two ADP-ribosylation factor 1 GTPase-activating proteins from rat liver

Paul A. RANDAZZO

Laboratory of Biological Chemistry, Division of Basic Sciences, National Cancer Institute, Bldg. 37, Rm 5D-02, 37 Convent Dr MSC 4255, Bethesda, MD 20892-4255, U.S.A.

ADP-ribosylation factor 1 (ARF1) is a 21 kDa GTP-binding protein that regulates multiple steps in membrane traffic. Here, two ARF1 GTPase-activating proteins (GAPs) from rat liver were resolved. The GAPs were antigenically distinct. One reacted with a polyclonal antibody raised against the GAP catalytic peptide previously purified by Makler et al. [Makler, Cukierman, Rotman, Admon and Cassel (1995) J. Biol. Chem. **270**, 5232–5237], and here is referred to as GAP1. The other GAP (GAP2) did not react with the antibody. These GAPs differed in phospholipid dependencies. GAP1 was activated 3–7-fold by the acid phospholipids phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidic acid (PA) and phosphatidylserine (PS). In contrast, GAP2 was stimulated 20–40-fold by PIP_2. PA and PS had no effect by themselves but PA increased GAP2 activity in the presence of PIP_2. The GAPs were otherwise similar in activity. In the presence of phosphoinositides, the K_m of GAP1 for ARF1–GTP was estimated to be $8.1 \pm 1.6\ \mu$M and the dissociation constant for ARF1–guanosine 5′,3-*O*-(thio)triphosphate (GTP[S]) was $7.4 \pm 2.2\ \mu$M. GAP2 was similar with a K_m for ARF1–GTP of $5.4 \pm 1.2\ \mu$M and a dissociation constant for ARF1–GTP[S] of $4.8 \pm 0.3\ \mu$M. Similarly, no differences were found in substrate preferences. Both GAP1 and GAP2 used ARF1 and ARF5 as substrates but not ARF6 or ARF-like protein-2. The potential role of multiple ARF GAPs in the independent regulation of ARF at specific steps in membrane traffic is discussed.

INTRODUCTION

ADP-ribosylation factors (ARFs) are a family of 21 kDa GTP-binding proteins that regulate membrane traffic [1–4]. Members of the family include the ARF proteins and the ARF-like proteins (Arls) [5–7]. The ARF proteins can be further divided by structural criteria into three classes [7]. Multiple members of the family occur within an organism. For instance, five ARFs have been identified in humans [6] and three ARFs in *Saccharomyces cerevisiae* [8,9]. Individual members of the family are highly conserved between species [6,7]. ARF was first identified and purified as a cofactor for cholera toxin-catalysed ADP-ribosylation of the heterotrimeric GTP-binding protein Gs [10,11]. Subsequently, genetic studies in *S. cerevisiae* revealed a role for ARF in membrane traffic [8]. Studies both *in vitro* and *in vivo* indicate that regulation of membrane traffic is also ARF's physiological function in mammalian cells [1,3,4,12].

Membrane traffic is regulated by coupling the binding and hydrolysis of GTP by ARF to the assembly and disassembly of protein coats on transport vesicles [1–3,12,13]. In eukaryotes, membrane traffic is mediated by vesicles that bud from a donor membrane and fuse with an acceptor membrane. The budding is driven by the assembly of a proteinaceous coat that must be shed before fusion. ADP-ribosylation factor 1 (ARF1)–GTP facilitates the assembly process by recruiting coat proteins to membranes [14–17]. Subsequent disassembly of the coat from the vesicle is triggered by the hydrolysis of GTP by ARF. Interference with GTP hydrolysis blocks vesicle fusion and leads to the accumulation of protein-coated vesicles both *in vitro* and *in vivo* [18–22].

ARF1 regulates membrane traffic at multiple sites within the cell. ARF1 co-localizes with Golgi-associated proteins when examined by immunofluorescence and has been shown to act at the Golgi [8,18,23,24]. However, ARF1 action is not confined to this organelle. ARF1 has been purified as the guanosine 5′,3-*O*-(thio)triphosphate (GTP[S])-dependent inhibitor of both intra-Golgi transport [25] and nuclear vesicle fusion [26]. Furthermore, ARF1 has been shown to affect ER-to-Golgi transport [19,27] and endosome–endosome fusion [28]. Even within the Golgi, ARF1 recruits proteins of two distinct types of vesicular coats, coatomer [14,17] and clathrin assembly protein-1 [15,16].

The hydrolysis of GTP by ARF1 is specifically catalysed and is a potential means of site-specific regulation of ARF1. GTPase-activating proteins (GAPs) for ARF1 have been found in bovine brain [29] and in rat liver [30,31]. The two GAPs have distinct phosphoinositide dependencies [29,30] and have been speculated to be contained in distinct catalytic polypeptides. However, because these proteins were purified from different species and tissues, available reagents could not be used to test whether they were distinct proteins. In this study, two ARF1 GAP activities from a rat-liver homogenate were resolved. The two GAPs were found to have similar affinities for ARF1–GTP and substrate specificities; however, the activities were contained in antigenically distinct polypeptides and had different phospholipid dependencies. Thus at least two distinct proteins stimulate hydrolysis of GTP by ARF1.

EXPERIMENTAL

Materials

L-α-Dimyrisotylphosphatidylcholine, an acid phospholipid fraction from bovine brain (phosphoinositides, catalogue no. P-6023, in the text referred to as phosphoinositides), phosphatidylinositol 4,5-bisphosphate (PIP_2), ATP, GTP and GTP[S] were from Sigma (St. Louis, MO, U.S.A.). Pyruvate kinase and

Abbreviations used: ARF, ADP-ribosylation factor; ARF1, ADP-ribosylation factor 1; Arl, ARF-like protein; DTT, dithiothreitol; GTP[S], guanosine 5′,3-*O*-(thio)triphosphate; GAP, GTPase-activating protein; PA, phosphatidic acid; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine.

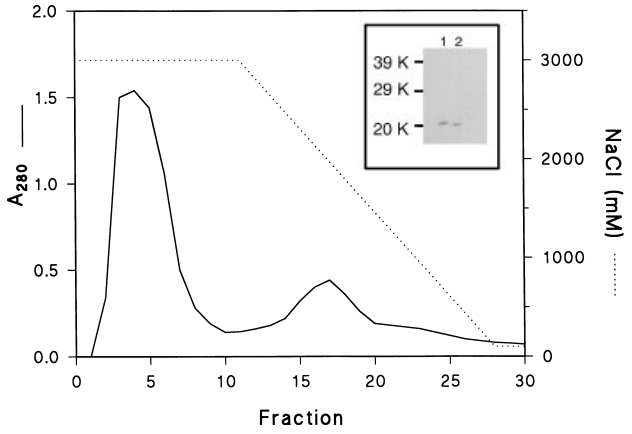

Figure 1 Separation of recombinant ARF1 from recombinant myristoylated ARF1

Approx. 5 mg of a mixture of myristoylated ARF1 and non-myristoylated ARF1 in 6 ml was applied to a 1 ml phenyl-Sepharose HP column and the column was developed as described in the Experimental section. Fractions 1–6 were collected during the load. Inset: Proteins from the first (lane 1) and second (lane 2) peaks were analysed by SDS/PAGE using a 10–20% (w/v) gradient gel. Molecular-mass markers are indicated on the left (K = kDa).

phospho(enol)pyruvate were from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). [α-^{32}P]GTP and [^{35}S]GTP[S] were purchased from DuPont/NEN (Boston, MA, U.S.A.). Frozen rat livers were obtained from Pel-Freez (Rogers, AR, U.S.A.). A rabbit polyclonal antibody raised against GAP1 [31] and recombinant GAP1 (containing residues 1–257) [31] were generously given by Dan Cassel (Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel).

Recombinant proteins

Recombinant, partially myristoylated ARF1 was prepared as previously described [32,33]. This material was typically 15% myristoylated. (The bovine nucleotide sequence for ARF1 was used. Although at the amino acid level, bovine and human ARF1 are 100% identical, use of the human nucleotide sequence in the dual transfection system used to modify ARF resulted in less than 2% myristoylation of ARF1.) The myristoylated protein was separated from the non-myristoylated protein by hydrophobic interaction chromatography. Recombinant protein (2–5 mg) was applied to a 1 ml phenyl-Sepharose HP column (Pharmacia Biotech, Piscataway, NJ, U.S.A.) that was then developed with a descending NaCl gradient from 3000 to 100 mM in 20 mM Tris/HCl (pH 8.0) containing 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT) run at 1 ml/min over 16 min at room temperature (Figure 1). Non-myristoylated ARF1 did not adsorb to the column under these conditions. The ARF1 protein eluting at 1500 mM NaCl was myristoylated based on three criteria: (i) faster migration than non-myristoylated ARF1 on SDS/PAGE (Figure 1, inset) [33]; (ii) co-elution with myristoylated ARF1 purified from bovine brain on reversed-phase HPLC performed as described in [33]; and (iii) a mass of 20778 ± 3 a.m.u. (the predicted mass of myristoylated ARF1 is 20775 a.m.u.). The protein was concentrated and desalted by ultrafiltration. Myristoylated ARF1 prepared by this method bound 0.58 ± 0.19 mol of GTP/mol of ARF1 ($n = 5$). Arl2 [6] was expressed as previously described [33]. The cDNA for hARF6 was generously given by Dr. Joel Moss (National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, U.S.A.) [7]. ARF6 was co-expressed with *N*-myristoyltransferase as described [33] and purified at room temperature by chromatography on DEAE–Sephacel followed by Q-Sepharose, both developed with linear gradients of 50–500 mM NaCl in 20 mM Tris/HCl (pH 8.0) containing 2 mM $MgCl_2$ and 1 mM DTT. ARF5 was expressed and purified as described before [34].

Assays

ARF GAP activity was determined using an assay that measures a single round of hydrolysis [29]. Phospholipids were solubilized in Triton X-100 and added as mixed micelles. The final concentration of Triton X-100 was 0.1% (v/v). For assaying column fractions, partially myristoylated recombinant ARF1 (1–3 μM) loaded with [α-^{32}P]GTP, as described in [29], was used as a substrate. For kinetic experiments, the substrate was fully myristoylated ARF1 loaded with [α-^{32}P]GTP by incubation, at a concentration of 200 μM, in 25 mM Hepes (pH 7.4) containing 100 mM NaCl, 2 mM ATP, 200–400 μM [α-^{32}P]GTP, 1.5 mM EDTA, 1 mM $MgCl_2$, 3 mM dimyristoylphosphatidylcholine, 0.1% (w/v) sodium cholate and 1 mM DTT, for 45 min at 30 °C. The amount of GTP binding to ARF was determined by filtering on nitrocellulose as described [35] and used to calculate the concentration of ARF1–GTP.

The affinity of GAP for ARF1–GTP[S] was determined by measuring the inhibition of GAP activity by ARF1–GTP[S]. Myristoylated ARF1 was loaded with GTP[S] by incubating for 60 min with 25 mM Hepes (pH 7.4) containing 100 mM NaCl, 400 μM GTP[S], 2 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 3 mM dimyristoylphosphatidylcholine and 0.1% sodium cholate. The concentration of ARF–GTP[S] was determined in a parallel reaction by measuring the binding of [^{35}S]GTP[S] to ARF [35].

As discussed [29], in most assays the concentration of ARF1–GTP was much less than the K_m. Under these conditions, substrate is consumed at a first-order rate equal to V_{max}/K_m. This rate is expressed as the fraction of ARF1–GTP hydrolysed/min.

Purification

Liver (40 g) was rapidly thawed and homogenized in 100 ml of a 20 mM Tris/HCl (pH 8.0) buffer containing 300 mM sucrose, 25 mM NaCl, 4 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 0.5 mM 1:10 phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotonin and 2 μg/ml leupeptin, by two 30 s bursts with a Polytron tissue homogenizer at a setting of 6. The homogenate was centrifuged at 23000 ***g*** for 30 min. The supernatant was collected and centrifuged at 100000 ***g*** for 60 min. The supernatant from the 100000 ***g*** centrifugation was frozen in a solid CO_2/ethanol bath and stored at −80 °C.

The 100000 ***g*** supernatant fraction (100 ml) was thawed and added to 2 vol. of 25 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and 1 mM DTT. Ammonium sulphate was added to a final concentration of 40% saturation. The mixture was incubated at 4 °C for 20 min and then centrifuged at 10000 ***g*** for 10 min. The pellets were resuspended in 40 ml of 25 mM Tris/HCl (pH 7.5) containing 1 mM DTT and 2.5 μg/ml leupeptin, clarified by centrifugation at 23000 ***g*** for 15 min and diluted with 60 ml of 25 mM Tris (pH 7.5) containing 1 mM DTT (solution A). The solution was applied to a 100 ml DEAE–Fractogel 650S (Tosohaas, PA, U.S.A.) run at 8 ml/min and 4 °C. The column was developed with three additional steps: 100 ml of solution A containing 100 mM NaCl and 1 μg/ml leupeptin (wash); 350 ml of solution A containing 155 mM NaCl; and 200 ml of solution A containing 255 mM NaCl.

The GAP activity in the material eluting with 100 mM NaCl was assayed in the presence of phosphoinositides for the re-

maining steps. The activity was applied at 4 °C to a 20 ml hydroxyapatite column (Biogel HTP, Bio-Rad, Hercules, CA, U.S.A.). The column was developed with a 140 ml gradient of 10–400 mM potassium phosphate (pH 7.0) in 100 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol and 20 % (v/v) glycerol run at 1 ml/min. The major peak was diluted with 2 vol. of 20 mM Tris (pH 8.0) containing 100 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol and 20 % glycerol, and applied to a 2 ml hydroxyapatite column equilibrated in 50 mM KP_i (pH 7.0) containing 100 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol and 20 % glycerol and run at 1 ml/min and 4 °C. The column was washed with 5–10 ml of 100 mM KP_i (pH 7.0) containing 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 20 % glycerol and 100 mM NaCl and was developed with a 70 ml linear gradient of 100–2000 mM NaCl. The material in the major peak was adjusted to a conductivity of 55 cm/m-ohm at room temperature with 4 M NaCl/20 % glycerol and applied to a 1 ml column of phenyl-Sepharose HP (Pharmacia Biotech). The column was developed at room temperature at 1 ml/min in a descending gradient of 2 M to 100 mM NaCl in 100 mM KP_i (pH 7.0), 1 mM $MgCl_2$, 1 mM β-mercaptoethanol and 20 % glycerol over 20 min. Fractions containing the activity were pooled and concentrated using a Centricon 30. This activity is referred to as GAP2. GAP activity eluting with 255 mM NaCl from the first DEAE column was purified as described by Makler et al. [30]. This activity is referred to as GAP1.

SDS/PAGE and immunoblotting

Proteins separated by SDS/PAGE were visualized either by Coomassie Blue or ProBlue Colloidal (Integrated Separation Systems, Natick, MA, U.S.A.) protein stains. Proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose in 25 mM Tris/192 mM glycine/20 % (v/v) methanol/0.01 % (w/v) SDS for 1 h at 100 V in a Mini Trans-Blot cell (Bio-Rad). Non-specific protein binding to the nitrocellulose was blocked by a 1 h incubation in Blotto [36]. The nitrocellulose was incubated with a rabbit polyclonal antibody raised against recombinant full-length ARF GAP1 from liver [31] (generously provided by Dan Cassel, Technion-Israel Institute of Technology) at a dilution of 1:2000 for 1 h at room temperature. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal (Amersham, Arlington Heights, IL, U.S.A.) and was incubated at a dilution of 1:1000 for 1 h at room temperature. The antibody complex was visualized by enhanced chemiluminescence (Amersham).

Miscellaneous

Protein concentrations were determined using the Bio-Rad dye binding assay and BSA as a standard. Lipids dissolved in chloroform/methanol were dried under a stream of nitrogen and solubilized in 1 % Triton X-100 at a concentration of 2–10 mg of phospholipid/ml. Electrospray ionization mass spectrometry was performed as described [37]. Other methods are as described [29]. All experiments have been performed on two separate preparations of each enzyme with similar results.

RESULTS

Two GAP activities from rat liver can be separated by anion exchange chromatography

Rat liver was homogenized and separated into soluble and particulate fractions by centrifugation at 100000 ***g***. Proteins from the soluble fraction were precipitated with ammonium sulphate, resolubilized and fractionated on an anion exchange column. Four fractions were collected: (1) material that did not adsorb to the column (flow through); (2) material that eluted with 100 mM NaCl; (3) material that eluted with 155 mM NaCl; and (4) material that eluted with 255 mM NaCl. GAP activity in the absence and presence of a preparation of phospholipids enriched in phosphoinositides (referred to as phosphoinositides) was compared for each (Table 1). When assayed in the absence of phosphoinositides, activity was found in three of the four fractions, with a flow-through of 100 mM NaCl and 255 mM NaCl. Phosphoinositides had no effect on the activity in the flow-through, they stimulated the activity eluting with 100 mM NaCl and they inhibited the activity eluting with 255 mM NaCl. Thus two distinct ARF GAP activities were separated. Each was further purified before characterization.

Table 1 Two ARF GAP activities resolved by anion exchange chromatography

Approx. 1 g of protein was applied to a DEAE–Fractogel column and the column was developed in four steps as described in the Experimental section. GAP activity was determined using ARF1–GTP at approx. 10 nM in the absence or presence of 1 mg/ml phosphoinositides. Activity is expressed as the percentage of total activity recovered in the presence of phosphoinositides and the data are means and range of two experiments. FT (flow-through) is the material that did not adsorb to the column; 100 mM, 155 mM and 255 mM are the materials that eluted with 100 mM, 155 mM and 255 mM NaCl.

	GAP activity (% of total)	
Column fraction	No addition	Phosphoinositides
FT	25 ± 3	36 ± 9
100 mM	21 ± 4	63 ± 9
155 mM	1 ± 1	0 ± 1
255 mM	14 ± 3	1 ± 1

Table 2 Purification of GAP2

GAP2 was purified as described in the Experimental section. S100 refers to the soluble fraction from the 100000 ***g*** centrifugation of the crude homogenate, ammonium sulphate is the solubilized ammonium sulphate precipitate of the protein in S100, DEAE is the material eluting from DEAE–Fractogel in 100 mM NaCl, hydroxyapatite 1 is the pool of activity from the hydroxyapatite column developed with a KP_i gradient and hydroxyapatite 2 is the pool of activity from the hydroxyapatite column developed with a NaCl gradient. Activity was determined using 15 nM ARF–GTP as substrate in the presence of 1 mg/ml crude phosphoinositides.

Step	Protein (mg)	Activity (nmol/min)	Specific activity (pmol/mg per min)	Fold purification	Recovery (%)
Homogenate	7280	4.9	0.67	1	100
S100	3000	3.8	1.26	1.9	78
Ammonium sulphate	880	2.5	2.8	4.3	51
DEAE	420	1.0	2.4	3.5	20
Hydroxyapatite 1	29	0.45	16	23	9
Hydroxyapatite 2	3.8	0.17	44	65	3.3
Phenyl-Sepharose	0.21	0.13	617	920	2.7

Purification of two GAPs

The GAP activity eluting from the DEAE column with 100 mM NaCl was further purified by three additional steps (Table 2). The material was first chromatographed on hydroxyapatite that was developed with an increasing KP_i gradient. Two broad overlapping peaks of activity were identified, one eluting between

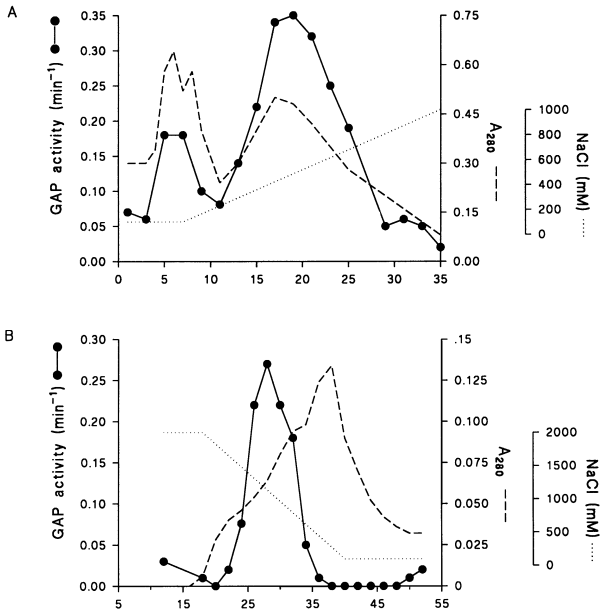

Figure 2 Purification of an ARF GAP activity eluting from DEAE–Fractogel in 100 mM NaCl

(**A**) Hydroxyapatite chromatography. ARF GAP activity (purified by hydroxyapatite with an increasing KP_i gradient) was diluted and chromatographed on a second hydroxyapatite column with an increasing NaCl gradient. (**B**) Hydrophobic interaction chromatography. The major peak of activity resolved by hydroxyapatite in Figure 3(A) was adsorbed to a phenyl-Sepharose column and eluted with a decreasing NaCl gradient.

Table 3 Purification of GAP1

GAP1 was purified as described in the Experimental section. S100 is the soluble fraction from the 100000 ***g*** centrifugation of the homogenate, ammonium sulphate is the solubilized ammonium sulphate precipitate of S100 protein, DEAE is the material eluting from DEAE–Fractogel in 255 mM NaCl, DEAE/urea is the pooled activity from the DEAE column developed in urea. Activity was determined using 15 nM ARF–GTP as a substrate.

Step	Protein (mg)	Activity (nmol/min)	Specific activity (pmol/mg per min)	Fold purification	Recovery (%)
Homogenate	7280	5.1	0.68	1	100
S100	3000	1.9	0.63	0.89	36
Ammonium sulphate	880	0.81	0.91	1.2	16
DEAE	75	0.41	5.5	7.4	8
DEAE/urea	1.6	0.052	33	49	1
Resource Q	0.065	0.0081	125	183	0.2

50 and 150 mM KP_i and the other between 150 and 250 mM. The latter peak of activity was pooled and adsorbed to a second hydroxyapatite column that was developed with an increasing gradient of NaCl. Two peaks were again observed (Figure 2A). A minor peak eluted early but most of the activity eluted with 400–600 mM NaCl. This latter activity was chromatographed on phenyl-Sepharose developed with a descending NaCl gradient. A single peak of activity eluted at approx. 1 M NaCl (Figure 2B). This material was purified approx. 900-fold over the starting homogenate and was used for further characterization.

The GAP activity eluting from the DEAE column with 255 mM NaCl was further purified with two additional chromatographic steps as described by Makler et al. [30] (Table 3). The material was applied to a second DEAE column that was

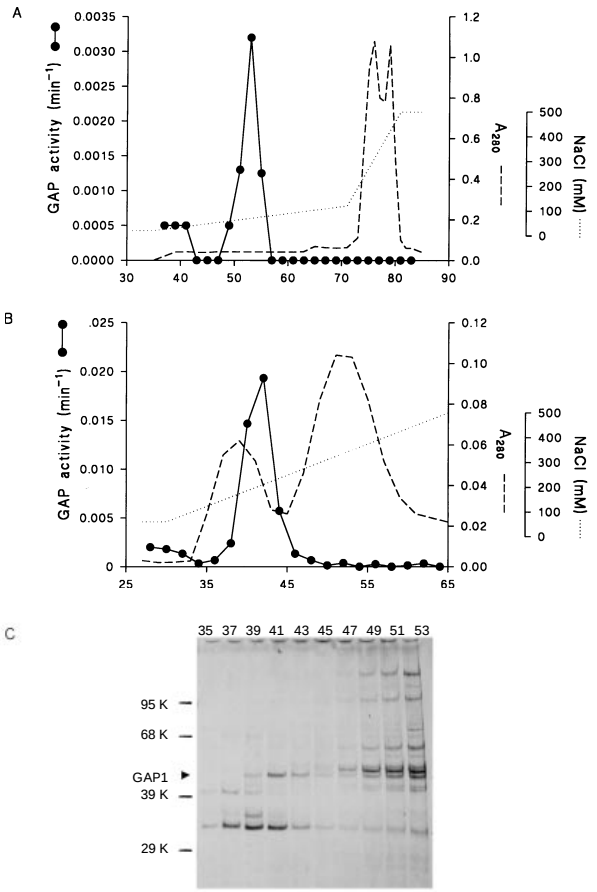

Figure 3 Purification of the GAP eluting from DEAE with 255 mM NaCl

(**A**) Anion exchange chromatography in the presence of urea. The material eluting at 255 mM NaCl from DEAE–Fractogel was diluted and applied to a second DEAE–Fractogel column. The column was developed in the presence of 5 M urea with an increasing gradient of NaCl as described in the Experimental section. (**B**) Fractionation on Resource Q. The activity resolved on the second DEAE column was adsorbed to a 1 ml Resource Q column that was then developed in the presence of 20% glycerol with an increasing gradient of NaCl. (**C**) SDS/PAGE analysis of proteins resolved by chromatography on Resource Q. Material from the indicated fractions of the Resource Q column was electrophoresed in SDS on a 10–20% polyacrylamide gel. Proteins were visualized by a Colloidal Blue stain as described in the Experimental section. The position at which GAP1 was expected to migrate is indicated by an arrow. Molecular mass markers are indicated on the left (K = kDa).

developed with an increasing NaCl gradient in the presence of 5 M urea. The activity eluted at 60 mM NaCl (Figure 3A). The pool of activity was then chromatographed on Resource Q and was developed with an increasing NaCl gradient in the presence of 20% glycerol. The activity eluted as a single peak (Figure 3B). The chromatographic behaviour was very similar to that reported by Makler et al. [30]. The proteins in the fractions containing activity were therefore analysed by SDS/PAGE. As Makler et al. [30] had found, the activity was found to co-elute with a polypeptide of approx. 48 kDa (Figure 3C). The activity purified through Resource Q was used for further characterization and, for this paper, is referred to as GAP1. The material purified through phenyl-Sepharose is referred to as GAP2.

GAP1 and GAP2 are distinct polypeptides

Proteins in the GAP1 and GAP2 preparations were fractionated by SDS/PAGE and visualized by Coomassie Blue protein

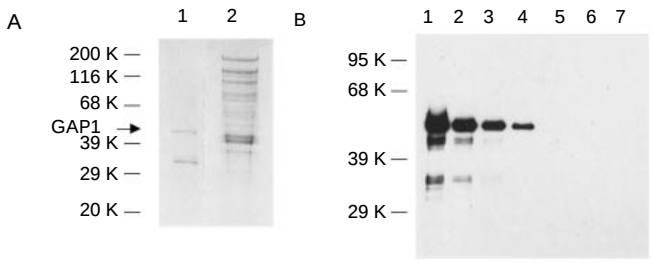

Figure 4 Activity in GAP1 and GAP2 is associated with different polypeptides

(**A**) GAP1 (1 μg; lane 1) and GAP2 (10 μg; lane 2) were electrophoresed in SDS on a 10–20% polyacrylamide gel. Proteins were visualized with Coomassie Blue protein dye. The position at which GAP1 was expected to migrate is indicated by an arrow. (**B**) GAP1 (0.4 μg in lane 1, 0.2 μg in lane 2, 0.1 μg in lane 3, 0.025 μg in lane 4) and GAP2 (0.75 μg in lane 5, 0.38 μg in lane 6, 0.19 μg in lane 7) were electrophoresed in SDS on a 10–20% polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose membrane was probed with an anti-GAP1 polyclonal antibody (generously provided by Dan Cassel, see the Experimental section) at a dilution of 1:2000. Enhanced chemiluminescence was used for detection. Molecular mass markers are indicated on the left (K = kDa).

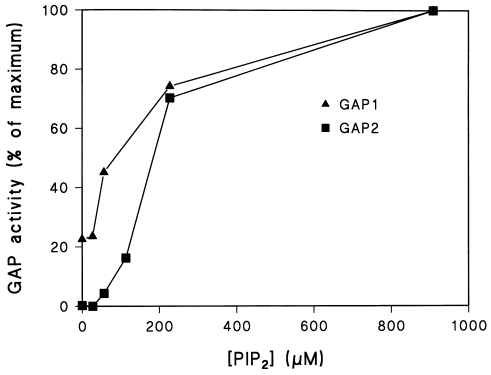

Figure 5 PIP_2 dependence of GAP1 and GAP2

The activity of GAP1 (triangles) and GAP2 (squares), both present at 2.6 μg/ml, was determined in the presence of the indicated concentrations of PIP_2. The data are expressed as the percentage of maximum observed activity, which was 0.031 min^{-1} for GAP1 and 0.092 min^{-1} for GAP2.

Table 4 Phospholipid dependence of two ARF GAPs

GAP activity in material eluting from the first DEAE column with 255 mM NaCl (crude GAP1, used at a concentration of 100 μg/ml in the assay), GAP1 (2.6 μg/ml) and GAP2 (2.6 μg/ml) was determined in the presence of the indicated phospholipids (PIs are the preparation of phospholipids enriched in phosphoinositides). The data are the average ± range for two experiments for crude GAP1 and purified GAP2 and the mean ± S.D. for three experiments for purified GAP1.

	Activity (fraction GTP hydrolysed/min)		
Phospholipids	Crude GAP1	GAP1	GAP2
No addition	0.016 ± 0.004	0.004 ± 0.001	0.011 ± 0.002
PIP_2, 90 μM		0.020 ± 0.005	0.027 ± 0.003
PIP_2, 450 μM	0.066 ± 0.009		
PA, 750 μM	0.073 ± 0.010	0.025 ± 0.003	0.006 ± 0.002
PA, 750 μM + PIP_2, 90 μM		0.027 ± 0.009	0.20 ± 0.004
PS, 750 μM	0.047 ± 0.005	0.020 ± 0.003	0.013 ± 0.001
PC, 750 μM		0.005 ± 0.001	0.007 ± 0.001
PIs, 1 mg/ml	0.031 ± 0.006	0.008 ± 0.001	0.052 ± 0.005

staining. As seen in Figure 4(A), lane 1, the GAP1 preparation contained two major bands, one at approx. 33 kDa and a second at approx. 48 kDa. The latter is presumably the polypeptide purified by Makler et al. [30] of molecular mass 49 kDa. Although GAP2 was still a complex mixture of polypeptides at this point in the purification (Figure 4A, lane 2), no band at 48 kDa was visualized. To exclude the possibility that a small amount of the 48 kDa protein might account for the GAP activity observed in the GAP2 preparation, GAP1 and GAP2 were fractionated by SDS/PAGE and transferred to nitrocellulose. The blots were probed with a rabbit polyclonal antibody raised by Cassel and colleagues [31] to the recombinant 49 kDa GAP from rat liver (Figure 4B). As anticipated, GAP1 contained the antigen. The smallest amount of material blotted gave a robust signal at 48–49 kDa. In contrast, no signal was detected among the GAP2 proteins even with 30-fold more GAP2 than GAP1 blotted (cf. lanes 4 and 5). Therefore the catalytic activity of GAP1 is contained in the same polypeptide purified by Makler et al. [30] and GAP2 is contained in a different polypeptide.

Stimulation of GAP1 and GAP2 by phospholipids

The effects of phosphoinositides on the purified preparations of GAP1 and GAP2 were re-examined. In the early steps of the purification, phosphoinositides stimulated GAP2 but inhibited GAP1. However, both GAPs have been reported to be stimulated by phosphoinositides [29,30]. Consistent with the previous reports, the purified GAPs were stimulated by a crude mixture of phosphoinositides (Table 4) and PIP_2 (Figure 5). The fraction from the first DEAE column containing GAP1 (labelled crude GAP1 in Table 4), which had been inhibited by phosphoinositides before freezing, was also activated by phosphoinositides after a single freeze–thaw cycle.

Although both GAP1 and GAP2 were activated by phosphoinositides, the dependencies were distinct. The activity of the crude and purified GAP1 was enhanced by all acid phospholipids tested including phosphatidic acid (PA) and phosphatidylserine (PS; Table 4). PIP_2 plus PA was no more effective than either phospholipid alone (Table 4). The concentration of PIP_2 for a half-maximal effect was 50–100 μM (Figure 5). In contrast, in the absence of other lipids, neither PS nor PA enhanced GAP2 activity (Table 4). As seen for bovine brain GAP [29], PA stimulated activity in the presence of 90 μM PIP_2 (Table 4). PIP_2, in the absence of a second phospholipid, enhanced activity with a half-maximal effect occurring at 100–200 μM PIP_2 (Figure 5). Phosphatidylcholine (PC) alone had no detectable effect on either GAP (Table 4).

Substrate specificities of GAP1 and GAP2

These two proteins with ARF GAP activity might differ in their abilities to discriminate between members of the ARF family of proteins. If this were the case, the GAPs could be expected to have different affinities for ARF1–GTP. To test this, the dependence of the rate on ARF1–GTP concentration was determined for each GAP (Figure 6). GAP2-induced hydrolysis of GTP on ARF was found to be saturable with a K_m of 5.4 ± 1.2 μM for ARF1–GTP (Figure 6A). In the absence of phospholipid, the rate of GTP hydrolysis induced by GAP1 was linearly proportional to the ARF1–GTP concentration up to 8 μM; however, in the presence of phosphoinositides, GAP2-catalysed hydrolysis of GTP on ARF was saturable with an estimated K_m of

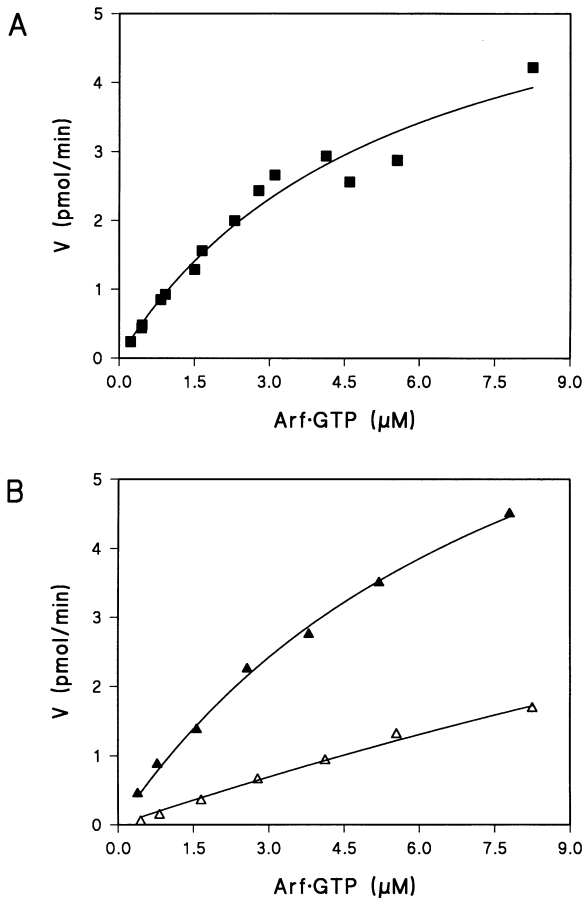

Figure 6 ARF1–GTP (Arf·GTP) concentration dependence

Myristoylated ARF1 was loaded with [α-^{32}P]GTP and incubated at the indicated concentrations with GAP in a total reaction volume of 25 μl. (**A**) GAP2. Incubations contained 2.6 μg/ml GAP2 and 1 mg/ml phosphoinositides. (**B**) GAP1. Incubations contained 5.2 μg/ml GAP1 and either no phospholipid (open triangles) or 1 mg/ml phosphoinositides (solid triangles).

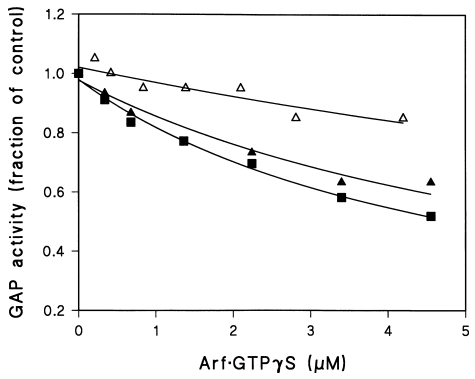

Figure 7 Inhibition of GAP by ARF1–GTP[S] (Arf·GTPγS)

Myristoylated ARF1–GTP (approx. 10 nM) was incubated with the indicated concentration of myristoylated ARF1–GTP[S] and GAP1 (triangles) or GAP2 (squares) in the presence (solid symbols) or absence (open triangles) of 1 mg/ml phosphoinositides.

8.1 ± 1.6 μM (Figure 6B). Thus based on saturation kinetics, GAP1 and GAP2 were similar to ARF1 GAPs.

The inhibition of GAP1 and GAP2 by ARF1–GTP[S] was examined as an independent corroboration of the K_m values. With a non-hydrolysable analogue of GTP bound, ARF1 should act as an inhibitor with a K_i approximating the K_m. This was found to be the case (Figure 7). GAP2 was inhibited with a K_i of 4.8 ± 0.3 μM. In the absence and presence of phosphoinositides, K_i values for GAP1 were estimated to be 18 ± 4 and 7.4 ± 2.2. Thus GAP1 and GAP2 could not be distinguished on the basis of affinities for ARF1–GTP determined in the presence of phosphoinositides.

Table 5 Substrate specificity of GAP1 and GAP2

ARF5, ARF6 and Arl2 were loaded with [α-^{32}P]GTP and incubated with 1 mg/ml phosphoinositides and no added GAP, 6.5 μg/ml GAP2 or 13 μg/ml GAP1 for 8 min. Assays were performed in duplicate. The GAP activity is expressed as a percentage of the average GAP activity using ARF1 as a substrate, which was 0.12 min^{-1} and 0.032 min^{-1} for GAP2 and GAP1. The mean $\pm$ range is shown.

	GAP activity (% of activity using ARF1 as substrate)	
Substrate	GAP1	GAP2
ARF5	86 ± 9	94 ± 7
ARF6	7 ± 5	1 ± 1
Arl2	4 ± 4	0.5 ± 1

The possibility that GAP1 and GAP2 had different substrate specificities was further examined by determining if ARF5 (a class 2 ARF), ARF6 (a class 3 ARF) or Arl2 was used as substrate by either GAP (Table 5). Similarly to ARF1, ARF5, ARF6 or Arl2 had no detectable intrinsic GTPase activity (< 0.002 min^{-1}). Both GAP1 and GAP2 stimulated hydrolysis of GTP on ARF5 at rates very similar to those observed with ARF1 as a substrate (Table 5). For GAP1, the activity using ARF5 as a substrate is not due to a contaminating polypeptide; recombinant GAP1 also stimulated hydrolysis of GTP by ARF5 at $95(\pm 12)\%$, the rate seen with ARF1. In contrast, neither GAP1 nor GAP2 increased the rate of GTP hydrolysis by ARF6 or Arl2. Thus although these two GAP activities were contained in distinct proteins, they could not be distinguished on the basis of either substrate specificities or affinities for ARF1–GTP.

DISCUSSION

ARF1 regulates membrane traffic at a number of distinct intracellular sites by cycling between GTP and GDP bound states. In this study, two antigenically distinct ARF1 GAPs, GAP1 and GAP2, were identified in a single tissue. Although the GAP activities were similar in their kinetic properties, each GAP exhibited differences in phospholipid requirements that could determine the site of action and, thereby, provide site-specific regulation of ARF1.

The two GAPs were separated by fractionation of a crude liver homogenate on an anion exchange column. The proteins were further purified before detailed analysis. One of the GAPs had been previously purified by Makler et al. [30] and is referred to as GAP1. In the other GAP preparation (GAP2), no polypeptide reacted with the polyclonal antibody to GAP1. The affinities of GAP1 and GAP2 for ARF1–GTP were similar and consistent with the expected intracellular concentration of ARF1 (which comprises 0.1–1 % of cellular proteins [5]), and both GAPs had a similar ability to discriminate between ARF family members using ARF1 and ARF5 as substrates but not ARF6 or Arl2.

Thus two distinct proteins are shown to function similarly *in vitro* as negative regulators of class 1 and class 2 ARFs.

The lipid dependencies provide a dramatic difference between GAP1 and GAP2. GAP2 had a specific PIP_2 dependence. In the absence of PIP_2, other acid phospholipids had no effect on activity. PA reduced the concentration of PIP_2 required for activation. In contrast, all acid phospholipids tested increased the activity of GAP1, and no positive interaction between PA and PIP_2 was observed.

GAP1 and GAP2 were further distinguished by the effects of phosphoinositides on activity in fractions from the first DEAE column. In fractions containing GAP2, activity was enhanced by a lipid fraction enriched in PIP_2, whereas in fractions containing GAP1, activity was inhibited. After either a single freeze–thaw cycle or chromatography in urea, the inhibition by phosphoinositides was no longer detected. Further work will determine whether this labile inhibitory factor is of relevance. However, this phenomenon is of interest in that it can explain why both groups who had previously studied ARF GAPs found only one GAP. By assaying in the absence of phosphoinositides, as in [30], GAP1 would be more easily detected. Assaying in the presence of phosphoinositides, as in [29], would suppress GAP1 activity, and only GAP2 would be detected.

The different lipid dependencies may determine the site of action of the GAPs. Multiple GAPs for a single monomeric GTP-binding protein is well precedented. This was first reported for ras with rasGAPp120 and NF1 [38]. Similarly, a number of GAPs have been found for Rho family GTP-binding proteins [39]. The function of these multiple GAPs is not clear but their activity has been proposed to be site-specific [39]. Similarly, there may be multiple ARF1 GAPs with distinct sites of action. For example, GAP1 may act at the relatively PIP_2-poor Golgi, where it has been localized by immunofluorescent studies [31], whereas GAP2 may function at the relatively PIP_2-rich plasma membranes. Both GAPs were found to use ARF1 and ARF5, but neither ARF6 nor Arl2, as substrates. This pattern of ARF interactions is different from that seen for other *in vitro* assays of ARF activity. Members of all three classes of ARF activate phospholipase D [32], the only effector of ARF yet identified [40,41]. In contrast, class 1 and 2 ARFs have different sensitivities to brefeldin A [43], an agent thought to inhibit ARF-activating protein [23,42]. Given that two activities that regulate ARF are able to distinguish between members of the ARF family, but the one available effector assay is not, ARF family members may have common effectors but be differentially regulated. In this way, protein-coat assembly may be similar to vesicle fusion in which there is a common effector, N-ethylmaleimide-sensitive fusion protein (NSF), common to different sites, that interacts with the site-specific soluble NSF attachment proteins (SNAPs) and SNAP receptors (SNAREs) [44,13].

I thank Drs. John K. Northup, Ronald Felsted, Julie Donaldson and Ulrike Kleuser for helpful discussions, Dr. Henry M. Fales for performing the electrospray ionization mass spectrometry analysis, Ulrike Kleuser and Sandor Berghoffer for preparing recombinant proteins, Dan Cassel for anti-GAP antibody, recombinant GAP1 and advice, Joel Moss for the cDNA for ARF6 and Albert Fornace and the Division of Basic Sciences, NCI, for continued support.

REFERENCES

1 Rosenwald, A. G. and Kahn, R. A. (1994) in GTPase-Controlled Molecular Machines (Corda, D., Hamm, H. and Luini, A., eds.), pp. 143–155, Ares-Serono Symposium Publications, Rome
2 Schekman, R. and Orci, L. (1996) Science **271**, 1526–1533
3 Donaldson, J. G. and Klausner, R. D. (1994) Curr. Opin. Cell Biol. **6**, 527–532
4 Nuoffer, C. and Balch, W. E. (1994) Annu. Rev. Biochem. **63**, 949–990
5 Kahn, R. A. (1993) in GTPases in Biology, Vol. 1, (Dickey, B. F. and Birnbaumer, L., eds.), 1st edn., pp. 529–542, Springer-Verlag, Berlin
6 Clark, J., Moore, L., Krasinskas, A., Way, J., Battey, J., Tamkun, J. and Kahn, R. A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8952–8956
7 Tsuchiya, M., Price, S. R., Tsai, S.-C., Moss, J. and Vaughan, M. (1991) J. Biol. Chem. **266**, 2772–2777
8 Stearns, T., Willingham, M. C., Botstein, D. and Kahn, R. A. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1238–1242
9 Lee, F.-J. S., Stevens, L. A., Kao, Y. L., Moss, J. and Vaughan, M. (1994) J. Biol. Chem. **269**, 20931–20937
10 Schleifer, L. S., Kahn, R. A., Hanski, E., Northup, J. K., Sternweis, P. C. and Gilman, A. G. (1982) J. Biol. Chem. **257**, 20–23
11 Kahn, R. A. and Gilman, A. G. (1984) J. Biol. Chem. **259**, 6228–6234
12 Moss, J. and Vaughan, M. (1995) J. Biol. Chem. **270**, 12327–12330
13 Rothman, J. E. (1994) Nature (London) **372**, 55–63
14 Donaldson, J. G., Cassel, D., Kahn, R. A. and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6408–6412
15 Stamnes, M. A. and Rothman, J. E. (1993) Cell **73**, 999–1005
16 Traub, I. M., Ostrom, J. A. and Kornfeld, S. (1993) J. Cell Biol. **123**, 561–573
17 Palmer, D. J., Helms, J. B., Beckers, C. J. M., Orci, L. and Rothman, J. E. (1993) J. Biol. Chem. **268**, 12083–12089
18 Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R. A. and Rothman, J. E. (1991) Cell **67**, 239–253
19 Dascher, C. and Balch, W. E. (1994) J. Biol. Chem. **269**, 1437–1448
20 Zhang, C.-J., Rosenwald, A. G., Willingham, M. C., Skuntz, S., Clark, J. and Kahn, R. A. (1994) J. Cell Biol. **124**, 289–300
21 Teal, S. B., Hsu, V. W., Peters, P. J., Klausner, R. D. and Donaldson, J. G. (1994) J. Biol. Chem. **269**, 3135–3138
22 Tanigawa, G., Orci, L., Amherd, M., Ravazzola, M., Helms, J. B. and Rothman, J. E. (1993) J. Cell Biol. **123**, 1365–1371
23 Donaldson, J. G., Finazzi, D. and Klausner, R. D. (1992) Nature (London) **360**, 350–352
24 Kahn, R. A., Randazzo, P., Serafini, T., Weiss, O., Rulka, C., Clark, J., Amherdt, M., Roller, P., Orci, L. and Rothman, J. E. (1992) J. Biol. Chem. **267**, 13039–13046
25 Taylor, T. C., Kahn, R. A. and Melancon, P. (1992) Cell **70**, 69–79
26 Boman, A. L., Taylor, T. C., Melancon, P. and Wilson, K. L. (1992) Nature (London) **358**, 512–514
27 Balch, W. E., Kahn, R. A. and Schwaninger, R. (1992) J. Biol. Chem. **267**, 13053–13061
28 Lenhard, J. M., Kahn, R. A. and Stahl, P. D. (1992) J. Biol. Chem. **267**, 13047–13052
29 Randazzo, P. A. and Kahn, R. A. (1994) J. Biol. Chem. **269**, 10758–10763
30 Makler, V., Cukierman, E., Rotman, M., Admon, A. and Cassel, D. (1995) J. Biol. Chem. **270**, 5232–5237
31 Cukierman, E., Huber, I., Rotman, M. and Cassel, D. (1995) Science **270**, 1999–2002
32 Randazzo, P. A., Weiss, O. and Kahn, R. A. (1992) Methods Enzymol. **219**, 362–369
33 Randazzo, P. A. and Kahn, R. A. (1995) Methods Enzymol. **250**, 394–405
34 Massenburg, D., Han, J. S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J. and Vaughan, M. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 11718–11722
35 Northup, J. K., Smigel, M. D. and Gilman, A. G. (1982) J. Biol. Chem. **257**, 11416–11423
36 Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 496–497, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37 Randazzo, P. A., Terui, T., Sturch, S., Fales, H. M., Ferrige, A. G. and Kahn, R. A. (1995) J. Biol. Chem. **270**, 14809–14825
38 Lowy, D. R. and Willumsen, B. M. (1993) Annu. Rev. Biochem. **62**, 851–891
39 Lamarche, N. and Hall, A. (1994) Trends Genet. **10**, 436–440
40 Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) Cell **75**, 1137–1144
41 Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O. and Hsuan, J. J. (1994) Science **263**, 523–526
42 Helms, J. B. and Rothman, J. E. (1992) Nature (London) **360**, 352–354
43 Tsai, S. C., Adamik, R., Haun, R. S., Moss, J. and Vaughan, M. (1993) J. Biol. Chem. **268**, 10820–10825
44 Melmon, I. (1995) Cell **82**, 869–872

Received 5 September 1996/13 January 1997; accepted 20 January 1997