Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 305–309, January 1996
Biochemistry

Purification and characterization of a guanine nucleotide-exchange protein for ADP-ribosylation factor from spleen cytosol

SU-CHEN TSAI*, RONALD ADAMIK, JOEL MOSS, AND MARTHA VAUGHAN

Pulmonary/Critical Care Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by Martha Vaughan, September 22, 1995

ABSTRACT **ADP-ribosylation factors (ARFs) are 20-kDa guanine nucleotide-binding proteins and are active in the GTP-bound state and inactive with GDP bound. ARF·GTP has a critical role in vesicular transport in several cellular compartments. Conversion of ARF·GDP to ARF·GTP is promoted by a guanine nucleotide-exchange protein (GEP). We earlier reported the isolation from bovine brain cytosol of a 700-kDa protein complex containing GEP activity that was inhibited by brefeldin A (BFA). Partial purification yielded an ≈60-kDa BFA-insensitive GEP that enhanced binding of ARF1 and ARF3 to Golgi membranes. GEP has now been purified extensively from rat spleen cytosol in a BFA-insensitive, ≈55-kDa form. It activated class I ARFs (ARFs 1 and 3) that were N-terminally myristoylated, but not nonmyristoylated ARFs from class I, II, or III. GEP activity required $MgCl_2$. In the presence of 0.6–0.8 mM $MgCl_2$ and 1 mM EDTA, binding of guanosine 5′-[γ-[^{35}S]thio]triphosphate ([^{35}S]GTPγS) by ARF1 and ARF3 was equally high without and with GEP. At higher Mg^{2+} concentrations, binding without GEP was much lower; with 2–5 mM $MgCl_2$, GEP-stimulated binding was maximal. The rate of GDP binding was much less than that of GTPγS with and without GEP. Phospholipids were necessary for GEP activity; phosphatidylinositol was more effective than phosphatidylserine, and phosphatidic acid was less so. Other phospholipids tested were ineffective. Maximal effects required ≈200 μM phospholipid, with half-maximal activation at 15–20 μM. Release of bound [^{35}S]GTPγS from ARF3 required the presence of both GEP and unlabeled GTP or GTPγS; GDP was much less effective. This characterization of the striking effects of Mg^{2+} concentration and specific phospholipids on the purified BFA-insensitive ARF GEP should facilitate experiments to define its function in vesicular transport.**

ADP-ribosylation factors (ARFs) are 20-kDa guanine nucleotide-binding proteins (GTP-binding proteins) initially discovered as activators of cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ (stimulatory regulator of adenylyl cyclase) (1). They are activated when GTP or the nonhydrolyzable analogue guanosine 5′-[γ-thio]triphosphate (GTPγS), but not GDP or ATP, is bound. Nucleotide binding and activation are enhanced by certain phospholipids and detergents (2, 3). ARFs are ubiquitous in eukaryotic cells from *Giardia* to mammals (2, 3). Six known mammalian ARFs fall into three classes based on deduced amino acid sequences and gene structure (2–4). The N-terminal glycines, after removal of the initiating methionine, are myristoylated, and it has been suggested that ARF interaction with membranes and phospholipids involves the myristoyl group and N-terminal helical region (5–9).

ARF proteins have been implicated in vesicular membrane trafficking in several intracellular compartments, including endoplasmic reticulum, Golgi, endosomes, and nuclear envelope (10–14). Cytosolic (15) as well as particulate (10, 16, 17) guanine nucleotide-exchange proteins (GEPs) that accelerate or enhance GTP binding and, thereby, ARF activation have been described. In the soluble fraction from bovine brain, an ≈700-kDa, brefeldin A (BFA)-sensitive complex (15), which includes a GEP that enhanced binding of ARF1 and ARF3, but not ARF5, to Golgi membranes was identified (18). From it, an ≈60-kDa BFA-insensitive GEP which increased binding of GTPγS by ARFs 1 and 3 was partially purified (15). ARF activation was dependent on GEP concentration and required phosphatidylserine (PS). The purified GEP was very unstable, however.

It has been reported that in the presence of low concentrations of $MgCl_2$, certain phospholipids—e.g., dimyristoyl phosphatidylcholine (DMPC)/cholate, DMPC/cholate plus phosphatidylinositol 4,5-bisphosphate (PIP_2), or azolectin—can promote guanine nucleotide exchange and activate ARF (19–21). We have now extensively purified an ≈55-kDa GEP from rat spleen cytosol that was BFA-insensitive and, unlike the GEP in rat brain cytosol, was not associated with a large protein complex. As reported here, GEP activity was critically dependent on the concentration of $MgCl_2$ and specific phospholipids. GTPγS binding was clearly favored over GDP. In experiments with recombinant ARF proteins, class I (rather than class II or class III) ARFs were preferred substrates for the partially purified GEP, and myristoylation of the ARF N-terminal glycine was important.

MATERIALS AND METHODS

Materials. Phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), PIP_2, and phosphatidic acid (PA) were purchased from Sigma, and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) was from Boehringer Mannheim. Sources of other materials have been published (15, 18).

Purification of GEP from Rat Spleen Cytosol. Frozen rat spleen (160 g) was homogenized (Polytron; Brinkmann) in 640 ml of TENDS buffer (20 mM Tris, pH 8.0/1 mM EDTA/1 mM NaN_3/1 mM dithiothreitol/0.25 M sucrose) containing protease inhibitors (aprotinin, leupeptin, soybean trypsin inhibitor, and lima bean trypsin inhibitor, each at 1 μg/ml; 0.5 mM phenylmethanesulfonyl fluoride; 0.5 mM AEBSF; 0.5 mM 1,10-phenanthroline; and 1 mM benzamidine) (15). All operations were at 4°C. The homogenate was centrifuged (12,000 × *g*, 1 hr) and the supernatant was further centrifuged (175,000 × *g*, Beckman SW 41 rotor, 37,000 rpm, 1.5 hr). To the supernatant (555 ml, 13.6 g of protein), solid $(NH_4)_2SO_4$ was

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; ARF, ADP-ribosylation factor; MyrARF, myristoyl-ARF; rARF, recombinant ARF; BFA, brefeldin A; GDPβS, guanosine 5′-[β-thio]diphosphate; GEP, guanine nucleotide-exchange protein; GTPγS, guanosine 5′-[γ-thio]triphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP_2, PI 4,5-bisphosphate; PS, phosphatidylserine; DMPC, dimyristoyl PC.

*To whom reprint requests should be addressed at: Room 5N-307, Building 10, National Institutes of Health, Bethesda, MD 20892.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

added to 30% saturation while the pH was maintained at ≈7.8 by addition of 0.74 M NH_4OH. After 45 min at 4°C, the precipitate was removed at centrifugation (12,000 × g, 45 min). To the supernatant, solid $(NH_4)_2SO_4$ was added to 60% saturation. After 45 min at 4°C and centrifugation, the precipitate was dissolved in 130 ml of homogenization buffer, dialyzed overnight against 4 liters of TENDS with 0.5 mM phenylmethanesulfonyl fluoride and added sucrose to a final concentration of 0.5 M, and then centrifuged (175,000 × g, SW41, 37,000 rpm, 1.25 hr).

The clear supernatant was applied to a column [5 cm × 40.8 cm, column volume (V_t) = 800 ml] of DEAE-Sephacel equilibrated with TENDSMA (TENDS, pH 8.0, with 2 mM $MgCl_2$ and 0.5 mM AEBSF) and eluted with (*i*) 0.5 V_t of TENDSMA, (*ii*) 2.5 V_t of TENDSMA containing 30 mM NaCl, and (*iii*) a linear gradient of 30–250 mM NaCl in TENDSMA (1.5 V_t of 30 mM plus 1.5 V_t of 250 mM). To each fraction (10 ml) collected during the gradient elution, 10 μl of 0.5 M AEBSF was added. Fractions with GEP activity, eluted between 90 and 140 mM NaCl, were pooled (428 mg of protein in 580 ml); proteins were precipitated by 65% saturation with $(NH_4)_2SO_4$, pelleted by centrifugation (12,000 × g, 1 hr), dissolved in 50 ml of PENDSMA (20 mM potassium phosphate, pH 7.0/1 mM EDTA/1 mM NaN_3/1 mM dithiothreitol/0.25 M sucrose/2 mM $MgCl_2$/0.5 mM AEBSF), and dialyzed overnight against 4 liters of the same buffer with added sucrose to a final concentration of 0.5 M. After centrifugation (12,000 × g, 60 min), the supernatant (40 ml, 210 mg of protein) was applied to a second column (2 cm × 15.9 cm, V_t = 50 ml) of DEAE-Sephacel equilibrated with PENDSMA (pH 7.0), and eluted with 4 V_t of PENDSMA followed by a linear gradient of 0 to 350 mM NaCl in PENDSMA (2 V_t of 0 mM plus 2 V_t of 350 mM). GEP activity was separated into two peaks, the first in the wash and the second eluted in salt gradient fractions. Further purification of the former GEP is described in Table 1. Meaningful estimation of the relative amounts of activity in the two peaks was not possible because of the presence of components that interfered with the assay.

Peak I GEP (100 ml, 8.2 mg of protein) was adjusted to pH 8.0 with 0.74 M NH_4OH, applied to a column (1.5 cm × 14.1 cm, V_t = 25 ml) of hydroxylapatite equilibrated with TENDSMA plus 0.1 M NaCl, and eluted with (*i*) 1 V_t of equilibration buffer, (*ii*) 0.5 V_t of equilibration buffer with 30 mM potassium phosphate (pH 8.0), and (*iii*) a linear gradient of 30 to 300 mM potassium phosphate in equilibration buffer (2 V_t 30 mM plus 2 V_t 300 mM). GEP was eluted between 165 and 220 mM phosphate (24 ml, 2.8 mg of protein). To remove phosphate, the pooled fractions were applied to a column (2 cm × 38.2 cm, V_t = 120 ml) of Sephadex G-25 equilibrated and eluted with TENDSMA. The recovered GEP (30 ml, 2.8 mg) was applied to a column (V_t = 3 ml) of heparin-agarose equilibrated with TENDSMA, then washed and eluted with (*i*) 2 V_t of equilibration buffer, (*ii*) 5 V_t of equilibration buffer with 200 mM NaCl, (*iii*) 1 V_t of equilibration buffer with 250 mM NaCl, and (*iv*) a linear gradient of 250 mM to 450 mM NaCl in equilibration buffer (3 V_t of 250 mM plus 3 V_t of 450 mM). GEP (0.34 mg of protein in 9.5 ml), eluted between 360 and 450 mM NaCl, was applied to a column (V_t = 1 ml) of hydroxylapatite equilibrated with TENDSMA plus 20 mM NaCl, washed with equilibration buffer, and eluted with 250 mM phosphate in TENDSMA containing only 0.125 M sucrose.

GEP (4 ml) was concentrated (Speed-Vac; Savant) to 2.2 ml and applied to a column (1.2 cm × 90.8 cm, V_t = 102.6 ml) of Ultrogel AcA 34 equilibrated with TENDSMA plus 0.5 M NaCl. Active fractions were combined (12 ml, 70 μg of protein), concentrated 3-fold by Centricon-10 (Amicon) with membranes that had been soaked overnight at 4°C in 1 ml of TENDSMA plus 0.5 M NaCl with 100 μg of bovine serum albumin, and stored in portions at −20°C until used. The GEP at this stage was very unstable. Several different preparations of GEP were used for the experiments reported here.

Assay of GEP Activity. Enhancement of guanine nucleotide binding to ARF by GEP was quantified directly by using radiolabeled nucleotide or indirectly by assessing ARF activity in the cholera toxin ADP-ribosyltransferase assay (15). All incubations were carried out at 33–37°C.

Preparation of ARF Proteins. ARFs 1 and 3 were purified from bovine brain cytosol and stored in 100-μl portions at −20°C (22). Recombinant ARF proteins were prepared as reported (8).

Table 1. Purification of GEP from rat spleen cytosol

Purification step	Protein, mg	Specific activity, unit(s)*/μg	Total activity, units × 10^{-3}
DEAE, pH 8.0	428 (100%)	0.135	57.8 (100%)
DEAE, pH 7.0	8.2 (18.9%)	0.175	14.2 (24.6%)
Hydroxylapatite	2.8 (0.7%)	3.6	10.0 (17.3%)
Heparin-agarose	0.34 (0.08%)	30	10.3 (17.8%)
Ultrogel AcA 34	0.07 (0.016%)	33	2.3 (4.0%)

Samples (2–10 μl) of indicated fractions were incubated in a total volume of 50 μl containing 0.1 μg of ARF3, 250 μM PS, 4 mM $MgCl_2$, 20 μM GTPγS, and other additions as described in *Materials and Methods* for 40 min at 36°C before transfer to an ice bath. For the second step, 2 μg of activated cholera toxin A subunit, agmatine, [^{14}C]NAD and other components were added for incubation for 1 hr at 30°C before isolation of [^{14}C]ADP-ribosylagmatine for radioassay (15). GEP from DEAE-Sephacel I chromatography (DEAE, pH 8.0) stimulated ARF activity up to 3-fold; as purification progressed, maximal activation reached >5-fold. Values in parentheses give total protein or activity relative to that in the pool from DEAE-Sephacel, pH 8.0.

*One unit of GEP activity is the amount required to increase 3-fold the activity of 0.1 μg of ARF3 in the standard assay.

RESULTS AND DISCUSSION

GEP from rat spleen cytosol has been purified (Table 1) more extensively than that from rat brain cytosol reported earlier (15). The major portion of GEP activity in rat spleen cytosol was eluted after bovine serum albumin on Ultrogel AcA 34 chromatography and was not inhibited by BFA (data not shown). GEP activity could not be detected in crude cytosol, and the maximal activation of ARF demonstrable with preparations in the early stages of purification was ≈3-fold. With greater purification, maximal activation exceeded 5-fold. In 13 purified preparations, we consistently recovered ≈100 μg of protein (≈0.0006%) from ≈15 g of rat spleen cytosolic protein (160 g of spleen). A 55-kDa band on silver-stained gels after SDS/PAGE, which represented 25–40% of total protein, was believed to be GEP because of its correlation with GEP activity in column fractions. For activation of 0.1–0.3 μg of ARF1 or ARF3 in standard assays, an estimated 5–20 ng of GEP was used.

GEP enhanced binding of [^{35}S]GTPγS by native ARF3 and MyrARF3 200–300% (Table 2). The significance of the apparent GEP effect (≈100% increase) on rARF3 binding is unclear, as is that on MyrARF6. [^{35}S]GTPγS binding to MyrARF5, rARF5, or rARF6 was not increased by GEP. As a GEP substrate, ARF3 (class I) was clearly better than ARF5 (class II) or ARF6 (class III), and myristoylation of N-terminal glycine was important. These data are consistent with earlier observations (18) that only class I ARFs were activated by soluble accessory protein, a crude soluble protein fraction from brain. The GEP, which was prepared by using ARF3 as the assay substrate during purification, also activated native ARF1, but it is not proven that the same GEP activated both.

Guanine nucleotide binding to ARF is known to be favored at low concentrations of Mg^{2+} (19). Effects of $MgCl_2$ concentration on [^{35}S]GTPγS or [^3H]GDP binding by ARFs 1 and 3

Table 2. Effect of GEP on binding of GTPγS to myristoylated or nonmyristoylated ARF proteins

ARF	Protein, μg	[^{35}S]GTPγS bound, pmol − GEP	+ GEP	+ GEP / − GEP
ARF3	0.07	0.47	2.0	4.3
	0.14	0.84	3.1	3.7
MyrARF3	0.3	0.34	1.4	4.1
	0.6	0.74	2.2	3.0
rARF3	0.3	0.08	0.17	2.1
	0.6	0.19	0.35	1.8
MyrARF5	0.3	2.1	2.4	1.1
	0.6	4.0	4.0	1.0
rARF5	0.3	0.29	0.32	1.1
	0.6	0.58	0.62	1.1
MyrARF6	0.3	0.18	0.37	2.1
	0.6	0.38	0.70	1.8
rARF6	0.3	0.32	0.37	1.2
	0.6	0.58	0.81	1.4

ARF3 (0.07 or 0.14 μg protein) purified from bovine brain cytosol or rARF protein (0.3 or 0.6 μg), 4 μM [^{35}S]GTPγS, 250 μM PS, and 3 mM $MgCl_2$, and other components, with or without 3.5 μl of GEP, were incubated for 40 min at 36°C. All recombinant (rARF3, rARF5, rARF6) and myristoylated recombinant (MyrARF3, MyrARF5, MyrARF6) ARFs were essentially equally active in the cholera toxin assay. Based on mobility on SDS/PAGE, MyrARF3 was only ≈50% myristoylated, whereas MyrARF5 and MyrARF6 appeared to be totally modified.

with or without GEP are shown in Fig. 1. In the presence of 1 mM EDTA, addition of 0.6 mM $MgCl_2$ (ARF1) or 0.9 mM $MgCl_2$ (ARF3) increased GTPγS binding to the same extent with or without GEP. In the absence of GEP, higher concentrations of $MgCl_2$ decreased GTPγS binding to a level similar to that without added $MgCl_2$. In the presence of GEP, GTPγS binding remained high with added $MgCl_2$ up to 5 mM. At similar concentrations of $MgCl_2$, GEP also enhanced binding of GDP, but considerably less than that of GTPγS. Doubling the amount of GEP with 2.2 or 3.2 mM $MgCl_2$ increased binding to apparently maximal levels (Fig. 1).

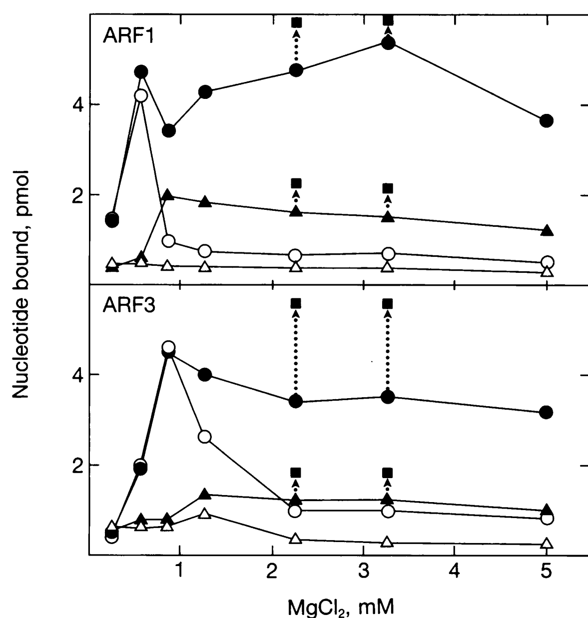

FIG. 1. Effect of $MgCl_2$ on binding of GTPγS and GDP by ARF1 and ARF3. Samples (0.2 μg) of ARF1 (*Upper*) or ARF3 (*Lower*) were incubated for 40 min at 35°C in a total volume of 50 μl containing 1 mM EDTA, 250 μM PS, 4 μM [^{35}S]GTPγS (○, ●) or [^{3}H]GDP (△, ▲), $MgCl_2$ as indicated, and 0 μl (○, △), 2.5 μl (●, ▲), or 5 μl (■) of GEP before radioassay of nucleotide bound to ARF.

With 4 mM $MgCl_2$ and PS, the rate of GTPγS binding was markedly increased by GEP and was minimal in its absence (Fig. 2). PIP_2, in the presence of which GTPγS binding was very slow with or without GEP, was much less effective than PS (Fig. 2). [^{3}H]GDP binding with GEP and PS at 1 mM or 4 mM $MgCl_2$ was much slower than GTPγS binding and continued at a relatively constant rate for at least 90 min (data not shown). Binding without GEP was faster with 1 mM $MgCl_2$ than with 4 mM $MgCl_2$, and GEP enhancement was greater with 4 mM $MgCl_2$ (data not shown).

As shown for GEP activity in bovine brain cytosol (15), PS markedly increased GEP activation of ARF when ARF activity was assayed by its effect on the ADP-ribosyltransferase activity of cholera toxin A subunit, with an apparent maximal effect at 200 μM (Fig. 3); it also increased ARF activity somewhat without GEP. In the absence of GEP, PIP_2 enhanced activity much more than did PS and addition of GEP had relatively little effect, consistent with the lack of effect of PIP_2 on GEP acceleration of GTPγS binding. PE and PC were without effect, with or without GEP. ARF activity was not enhanced by GEP in the absence of phospholipid. The increment in ARF activity observed with PIP_2 and PS in the absence of GEP was attributed to an effect of the phospholipid on ARF activation of cholera toxin A-subunit ADP-ribosyltransferase activity, rather than on the activation of ARF itself.

In the absence of GEP, individual phospholipids had markedly different effects on binding of GTPγS to ARF3, a few of these dramatically dependent on Mg^{2+} concentration (Table 3). With 0.85 mM $MgCl_2$ and 1 mM EDTA, GEP activity was essentially undetectable with or without phospholipids (Table 3). Of the lipids that increased GTPγS binding with or without GEP, DMPC/cholate, the most effective, has been used (20, 23) to "load" rARF1 with GTPγS or GDP. With DMPC/cholate, however, [^{35}S]GTPγS binding was unaffected by GEP and was much lower than the maximal binding achieved with GEP and 4 mM $MgCl_2$ (Table 3). With 4 mM $MgCl_2$, ARF binding of [^{35}S]GTPγS in the absence of GEP was enhanced similarly by DMPC/cholate, PI, and PA, and to a lesser extent by PS, PIP_2, PE, and PC, in that order. In the presence of GEP, PI was much more effective than PS, which was used in most experiments, and PA was less effective. The same phospholipids (PI > PS > PA) increased ARF binding of [^{3}H]GDP in

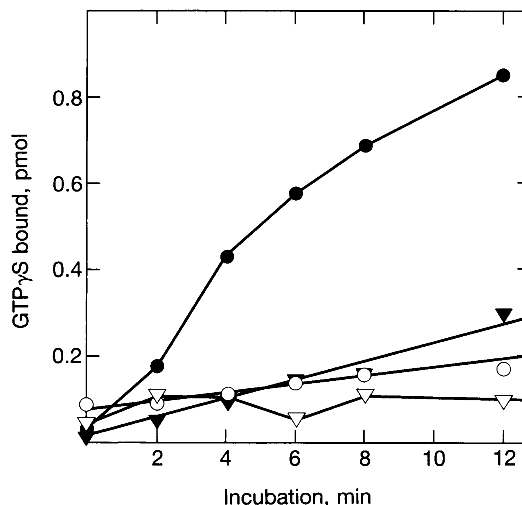

FIG. 2. Effect of GEP on GTPγS binding to ARF3. ARF3 (0.07 μg) was incubated at 33°C for the indicated time with 1 mM EDTA, 4 mM $MgCl_2$ (○, ●, ▽, ▼), 250 μM PS (○, ●) or 200 μM PIP_2 (▽, ▼), and 4 μM [^{35}S]GTPγS without (○, ▽) or with (●, ▼) GEP (2 μl) before radioassay of nucleotide bound to ARF.

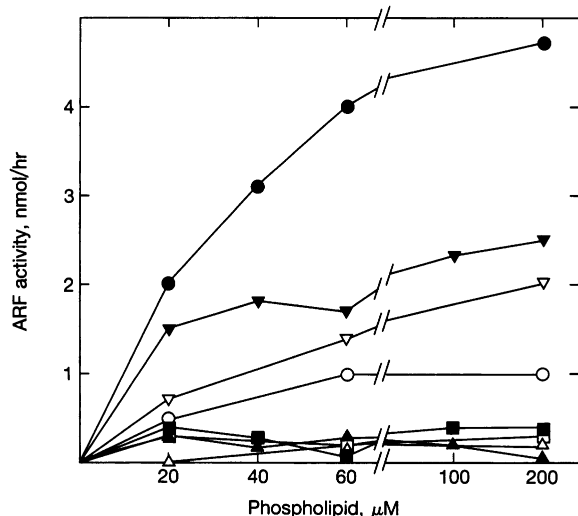

FIG. 3. Effects of phospholipids on ARF3 activity. ARF3 (0.08 μg) without (△, ○, □, ▽) or with (▲, ●, ■, ▼) 3 μl of GEP was incubated with 20 μM GTPγS, 4 mM $MgCl_2$, 1 mM EDTA, and phospholipid [PS (○, ●), PC (△, ▲), PIP_2 (▽, ▼), or PE (□, ■)] for 40 min at 37°C before assay of ARF activation (without further addition of phospholipid) of cholera toxin A-subunit-catalyzed ADP-ribosylagmatine formation. Activity of cholera toxin A subunit has been subtracted.

the presence of GEP and 4 mM $MgCl_2$ (data not shown). PIP_2 was much less effective, and PE and PC were ineffective (data not shown). Thus, in the presence of physiological concentrations of $MgCl_2$, GEP-catalyzed guanine nucleotide exchange was enhanced by three phospholipids that are important constituents of cellular membranes, although it has not been established that ARF activation in cells occurs in a membrane environment.

After binding of [^{35}S]GTPγS to ARF3 in the presence of 0.85 mM $MgCl_2$, 1.1 mM EDTA, and PS, effects of unlabeled GTPγS, GDP, or guanosine 5′-[β-thio]diphosphate (GDPβS) on its release in the presence of 4 mM $MgCl_2$ were assessed (Fig. 4). There was no release in the absence of GEP. When GEP was present, release in 30 min was similar with 1 μM GTPγS and 5 μM GDP. Effects of GTP were similar to those of GTPγS and ATP was without effect (data not shown). Doubling GEP increased release with 5 μM GTPγS or 10 μM GDP, and 15% release was observed with 10 μM GDPβS (Fig. 4). Incubation of ARF3·[^{35}S]GTPγS, prepared as described in the legend to Fig. 4, plus GEP and 4 mM $MgCl_2$ without added unlabeled nucleotide resulted in no loss of bound [^{35}S]GTPγS

Table 3. Effects of phospholipids and $MgCl_2$ on [^{35}S]GTPγS binding to ARF3 with or without GEP

	[^{35}S]GTPγS bound, pmol			
	0.85 mM $MgCl_2$		4 mM $MgCl_2$	
Phospholipid	− GEP	+ GEP	− GEP	+ GEP
None	0.34	0.28	0.43	0.51
PS	2.0	1.9	1.6	5.7
PI	2.2	1.9	2.0	8.8
PA	0.14	0.15	1.93	3.3
PIP_2	0.29	0.58	0.93	1.22
PC	0.49	0.47	0.56	0.58
PE	1.57	1.41	0.76	0.9
DMPC/cholate	3.6	3.4	2.1	2.1

ARF3 (0.2 μg), 1 mM EDTA, 4 μM [^{35}S]GTPγS, 200 μM phospholipid (except for 3 mM DMPC with 0.1% sodium cholate), with or without GEP (5 μl) and with $MgCl_2$ as indicated were incubated for 40 min at 36°C before collection of ARF·[^{35}S]GTPγS on nitrocellulose for radioassay.

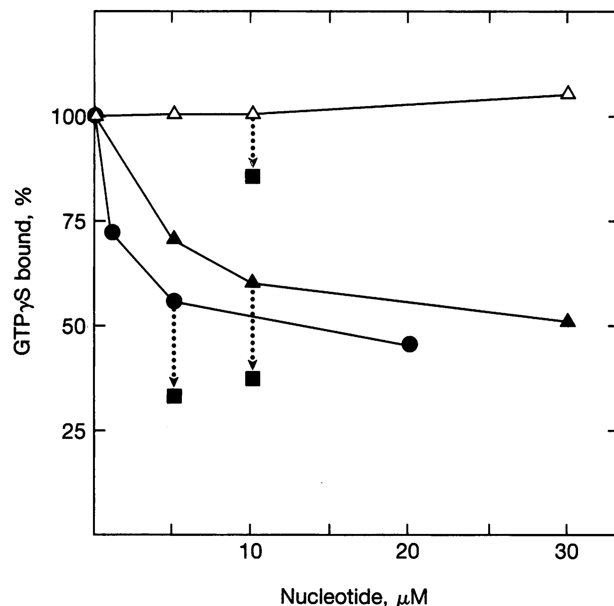

FIG. 4. Effect of nucleotides on release of bound GTPγS from ARF3. ARF3 (0.15 μg) was first incubated with 0.87 mM $MgCl_2$, 1.1 mM EDTA, 250 μM PS, and 1 μM [^{35}S]GTPγS for 30 min at 35°C, then cooled in an ice bath before addition of extra 4 mM $MgCl_2$ and unlabeled GTPγS (●), GDP (▲), or GDPβS (△) to the indicated concentrations, without or with 3 μl (●, △, ▲) or 6 μl (■) of GEP and further incubation for 30 min at 35°C (total volume, 50 μl). The 100% point (1.95–2.2 pmol of [^{35}S]GTPγS·ARF3) is the mean of values (n = 36) from samples incubated without GEP but with unlabeled nucleotide and 4 mM $MgCl_2$.

(Fig. 5). With 1 mM GTPγS, release was almost twice as fast as it was with 1 mM GDP (Fig. 5). Thus, measurable release (in 30 min) of bound [^{35}S]GTPγS from ARF3 in the presence of PS and 4 mM $MgCl_2$ required both GEP and unlabeled nucleotide. GTPγS or GTP was clearly more effective than GDP or GDPβS in this regard. This was also observed for the exchange of guanine nucleotide bound to *Saccharomyces cerevisiae* Ras2p catalyzed by Cdc25p, its guanine nucleotide

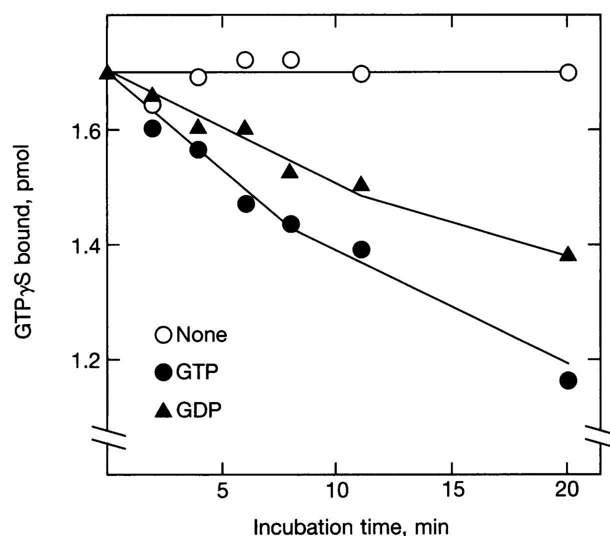

FIG. 5. Time course of release of bound GTPγS from ARF3. ARF3 (0.15 μg) was first incubated with 0.85 mM $MgCl_2$, 1 mM EDTA, 250 μM PS, and 1 μM [^{35}S]GTPγS for 30 min at 33°C, then cooled in an ice bath before addition of 3 μl of GEP and 4 mM $MgCl_2$, without (○) or with 1 mM GTPγS (●) or GDP (▲), and further incubated at 33°C for the time indicated (total volume, 50 μl).

exchange factor, as reported by Haney and Broach (24). Their detailed studies led to the conclusion that Cdc25p accelerates exchange by stabilizing the nucleotide-free Ras2p, and its corollary that Cdc25p interacts with a region of Ras2p, the conformation of which is independent of the identity of the bound nucleotide. The findings with GEP and class I ARFs reported here are seemingly consistent with a similar mechanism, but more extensive data are required to support this conclusion.

GEP is a part of the ARF regulatory cycle in which a GTPase-activating protein, ARF·GAP, catalyzes hydrolysis of ARF-bound GTP to yield inactive ARF·GDP, the substrate for GEP. The activity of ARF·GAP purified from rat liver cytosol (25) was enhanced by PI or PIP_2. It was reported that PA potentiated the effect of low concentrations of PIP_2 on ARF·GAP extracted from bovine brain membranes (26). PIP_2 was also important for ARF activation of phospholipase D (27), whose participation in a positive feedback loop involved in vesicle fusion at the target membrane has been postulated (28). The purified GEP, apparently specific for class I ARF activation, like that previously identified in the brain soluble accessory protein fraction (18), clearly preferred myristoylated over nonmyristoylated ARF as substrate. GAP isolated from membranes, however, accelerated GTP hydrolysis by both myristoylated and nonmyristoylated rARF; i.e., N-terminal myristoylation of ARF seemed less important for GAP activity than for GEP activity. This may be related to the requirement for acylation for effective association of cytosolic ARF with its site of membrane binding, for which the formation of ARF·GTP (catalyzed by GEP) is critical, whereas GAP presumably acts on ARF·GTP that is already in a membrane environment. In the cell, however, GEP may also be somehow associated with membranes, although apparently soluble after homogenization.

We are grateful to Dr. Randy Haun for preparation of myristoylated and nonmyristoylated recombinant ARFs. We thank Mrs. Carol Kosh for expert secretarial assistance.

1. Schleifer, L. S., Kahn, R. A., Hanski, E., Northup, J. K., Sternweis, P. C. & Gilman, A. G. (1982) *J. Biol. Chem.* **257,** 20–23.
2. Moss, J. & Vaughan, M. (1993) in *Handbook of Experimental Pharmacology*, eds. Dickey, B. F. & Birnbaumer, L. (Springer, Berlin), Vol. 108, pp. 543–559.
3. Moss, J. & Vaughan, M. (1995) *J. Biol. Chem.* **270,** 12327–12330.
4. Tsuchiya, M., Price, S. R., Tsai, S.-C., Moss, J. & Vaughan, M. (1991) *J. Biol. Chem.* **266,** 2772–2777.
5. Kahn, R. A., Goddard, C. & Newkirk, M. (1988) *J. Biol. Chem.* **263,** 8282–8287.
6. Kahn, R. A., Randazzo, P., Serafini, T., Weiss, O., Rulka, C., Clark, J., Amherdt, M., Roller, P., Orci, L. & Rothman, J. E. (1992) *J. Biol. Chem.* **267,** 13039–13046.
7. Walker, M. W., Bobak, D. A., Tsai, S.-C., Moss, J. & Vaughan, M. (1992) *J. Biol. Chem.* **267,** 3230–3235.
8. Haun, R. S., Tsai, S.-C., Adamik, R., Moss, J. & Vaughan, M. (1993) *J. Biol. Chem.* **268,** 7064–7068.
9. Randazzo, P. A., Terui, T., Sturch, S., Fales, H. M., Ferrige, A. G. & Kahn, R. A. (1995) *J. Biol. Chem.* **270,** 14809–14815.
10. Randazzo, P., Yang, Y. C., Rulka, C. & Kahn, R. A. (1993) *J. Biol. Chem.* **268,** 9555–9563.
11. Balch, W. E., Kahn, R. A. & Schwaninger, R. (1992) *J. Biol. Chem.* **267,** 13053–13061.
12. Boman, A. L., Taylor, T. C., Melançon, P. & Wilson, K. L. (1992) *Nature (London)* **358,** 512–514.
13. Lenhard, J. M., Kahn, R. A. & Stahl, P. D. (1992) *J. Biol. Chem.* **267,** 13047–13052.
14. Zeuzem, S., Feick, P., Zimmerman, P., Haase, W., Kahn, R. A. & Schulz, I. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6619–6623.
15. Tsai, S.-C., Adamik, R., Moss, J. & Vaughan, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3063–3066.
16. Donaldson, J. G., Finazzi, D. & Klausner, R. D. (1992) *Nature (London)* **360,** 350–352.
17. Helms, J. B. & Rothman, J. E. (1992) *Nature (London)* **360,** 352–354.
18. Tsai, S. C., Adamik, R., Haun, R. S., Moss, J. & Vaughan, M. (1993) *J. Biol. Chem.* **268,** 10820–10825.
19. Weiss, O., Holden, J., Rulka, C. & Kahn, R. A. (1989) *J. Biol. Chem.* **264,** 21066–21072.
20. Franco, M., Chardin, P., Chabre, M. & Paris, S. (1995) *J. Biol. Chem.* **270,** 1337–1341.
21. Terui, T., Kahn, R. A. & Randazzo, P. A. (1994) *J. Biol. Chem.* **269,** 28130–28135.
22. Tsai, S.-C., Noda, M., Adamik, R., Chang, P. P., Chen, H.-C., Moss, J. & Vaughan, M. (1988) *J. Biol. Chem.* **263,** 1768–1772.
23. Randazzo, P. A., Terui, T., Sturch, S. & Kahn, R. A. (1994) *J. Biol. Chem.* **269,** 29490–29494.
24. Haney, S. A. & Broach, J. R. (1994) *J. Biol. Chem.* **269,** 16541–16548.
25. Makler, V., Cukierman, E., Rothman, M., Admon, A. & Cassel, D. (1995) *J. Biol. Chem.* **270,** 5232–5237.
26. Randazzo, P. & Kahn, R. A. (1994) *J. Biol. Chem.* **269,** 10758–10763.
27. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. & Sternweis, P. C. (1993) *Cell* **75,** 1137–1144.
28. Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-S. & Cantley, L. C. (1994) *J. Biol. Chem.* **269,** 21403–21406.