The Protein Cofactor Necessary for ADP-ribosylation of G, by Cholera Toxin Is Itself a GTP Binding Protein*

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A membrane-bound protein cofactor (ARF) is required for the cholera toxin-dependent ADP-ribosylation of the stimulatory regulatory component (G*) of adenylate cyclase. Improved methods for the purification of ARF from bovine brain are described. ARF has a high-affinity binding site for guanine nucleotides. Binding of GTP or GTPγS to ARF is necessary for the activity of the cofactor; GDP-ARF does not support ADP-ribosylation of G*. Although the protein as purified contains stoichiometric amounts of GDP, GTPase activity of isolated ARF was not detected. Cholera toxin-dependent activation of adenylate cyclase thus requires two guanine nucleotide binding proteins.

A family of guanine nucleotide-binding regulatory proteins (G proteins) serves as membrane-bound information transducers, coupling activation of cell surface receptors to regulation of intracellular effectors (1, 2). G, and G are responsible for stimulatory and inhibitory control of adenylate cyclase, respectively; transducin activates a cyclic GMP-specific phosphodiesterase in the retina; the role of other G proteins is yet to be elucidated. These regulatory proteins share several structural and functional properties. Included among these is the fact that individual G proteins are substrates for bacterial toxins that catalyze the ADP-ribosylation of their a subunits. In the case of G, ADP-ribosylation is effected by cholera toxin (for review, see 3, 4).

Cholera toxin-dependent ADP-ribosylation of purified G requires lipid, NAD, GTP, and another membrane-bound protein cofactor termed ADP-ribosylation factor or ARF. The requirement for GTP has been demonstrated in membranes from several different sources (5–7), and we confirmed this requirement with purified components (8). In the absence of evidence for binding of GTP to ARF or cholera toxin, we concluded that binding of GTP to G was a prerequisite for its ADP-ribosylation. Gill and Meren (9) have suggested that two distinct guanine nucleotide binding sites are involved in the action of cholera toxin. These data and the development of new procedures for the purification of ARF, which allow production of milligram quantities of the factor in a more active form, prompted re-examination of the properties of the protein. ARF is indeed a guanine nucleotide-binding protein. Although the physiological role of ARF remains to be defined, these observations suggest that at least three GTP binding proteins, ARF, G, and G, may be involved in the regulation of adenylate cyclase activity.

MATERIALS AND METHODS

Purification of ARF—Bovine brain membranes were prepared as described by Sternweis and Robishaw (10), except that 0.5 mM phenylmethylsulfonyl fluoride was included. Extract was prepared from 12–15 g of membrane protein by incubation for 1 h at 4 °C in 2,000 ml of 20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1% sodium cholate, and 0.1 mM phenylmethylsulfonyl fluoride. Extracted membranes were removed by centrifugation at 95,000 × g for 1 h. Supernatants were pooled and activators (AMF or 6 mM MgCl2) were added at this point, as indicated. When activators were included they were added to all solutions used for chromatography on DEAE-Sepharose, Ultrogel AcA 44, and heptamidine-Sepharose.

The extract was applied to a DEAE-Sepharose column (5 × 60 cm), and proteins were eluted with a linear gradient (2 liters) of NaCl (0–250 mM) as described by Kahn and Gilman (8). The pooled DEAE peak was concentrated to approximately 30 ml by ultrafiltration using an Amicon PM 30 membrane and applied to an Ultrogel AcA 44 column as previously described (8). ARF eluted from the AcA 44 column with a Ks of 0.5. The pool of ARF activity from the AcA 44 column was diluted with TED containing 100 mM NaCl to a final concentration of 0.25% sodium cholate and applied to a 130-ml column (2.5 em) of heptamidine-Sepharose that had been equilibrated with 20 mM Tris-Cl (pH 8.0), 1% sodium cholate, and 0.1 mM phenylmethylsulfonyl fluoride. Extracted membranes were removed by centrifugation at 95,000 × g for 1 h. Supernatants were pooled and activators (AMF or 6 mM MgCl2) were added at this point, as indicated. When activators were included they were added to all solutions used for chromatography on DEAE-Sepharose, Ultrogel AcA 44, and heptamidine-Sepharose.

The extract was applied to a DEAE-Sepharose column (5 × 60 cm), and proteins were eluted with a linear gradient (2 liters) of NaCl (0–250 mM) as described by Kahn and Gilman (8). The pooled DEAE peak was concentrated to approximately 30 ml by ultrafiltration using an Amicon PM 30 membrane and applied to an Ultrogel AcA 44 column as previously described (8). ARF eluted from the AcA 44 column with a Ks of 0.5. The pool of ARF activity from the AcA 44 column was diluted with TED containing 100 mM NaCl to a final concentration of 0.25% sodium cholate and applied to a 130-ml column (2.5 cm) of heptamidine-Sepharose that had been equilibrated with 600 ml of TED containing 100 mM NaCl and 0.25% sodium cholate. After loading, the column was washed with 100 ml of equilibration buffer before development with a 600-ml linear gradient of 0.5–1.5% sodium cholate in TED. The flow rate was 50–70 ml/h, and 8.5-ml fractions were collected. The pool of ARF activity eluted between 0.8 and 1.0% cholate. The pooled heptamidine-Sepharose peak was concentrated to 1 ml by ultrafiltration using an Amicon PM 30 membrane. Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and elution of activity from the gel were performed as described (8).

Assay of ARF—ARF was assayed as described by Kahn and Gilman (8). In brief, purified rabbit liver G was incubated with activated cholera toxin, CTP, NAD, and ARF under conditions where the rate of ADP-ribosylation of G was linearly related to the amount of ARF in the sample. Covalent modification of G was then assessed by quantitation of the cholera toxin-dependent, GTP-stimulated adenylate cyclase activity after reconstitution of G into cyc (G-deeficient) membranes (11). The unit of ARF activity is expressed as pmol cyclic AMP/min/μg of G. (see Ref. 8).

Guanine Nucleotide Binding Assay—Binding of nucleotides to ARF was determined with a modification of the rapid filtration technique described by Norup et al. (12). Radioactivity retained by the filter was linearly related to the amount of protein applied. At least 70%...
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(78 ± 8%; n = 6) of bound nucleotide was retained by the filter under the conditions utilized, as compared to samples in which unbound ligand was removed by gel filtration on G-25 Sephadex. ARF was preincubated at 30 °C in either 20 mM Na Hepes (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 800 mM NaCl, 6 mM MgCl₂, 3 mM DMPC, 0.1% sodium cholate, and nucleotide, as described in the text. The binding reaction was stopped by the addition of 2 ml of ice-cold buffer (25 mM Tris-Cl, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol), followed by rapid filtration on 25-mm BA 85 nitrocellulose filters (Schleicher and Schuell). Filters were washed seven times with 2 ml of the same buffer, dried under a heat lamp, and counted in 8 ml of scintillation mixture. Standards for each assay were dried on filters and counted identically. The zero-time blank, caused by contamination of substrate with ³²P, was less than 0.2% of the total.

Purification of ARF by HPLC—ARF was purified through the heptanoyl-Sepharose stage, and the solution was concentrated to 1 ml. ARF was generally 90% pure at this point. Concentrated ARF was applied to two 300 × 7.5 mm Bio-Sil TSK-250 columns (Bio-Rad) in series, previously equilibrated in 50 mM Na Hepes (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol, and 6 mM MgCl₂. The columns were eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and assayed for protein, ARF, and guanine nucleotide binding.

Identification of Nucleotide Bound to ARF— Determination of the guanine nucleotide content of pure ARF was performed as described by Ferguson et al. (14). ARF was purified to homogeneity by HPLC and chromatographed on Sephadex G-25 in 10 mM potassium phosphate, pH 7.0, and a flow rate of 1.0 ml/min was used as described above. The ARF sample was washed with one column volume of equilibration buffer. Nucleotides were eluted with the following program of washes and gradients: 10–300 mM potassium phosphate over 1 min, 300 mM potassium phosphate for 2 min; 300–650 mM potassium phosphate over 20 min, and 650–700 mM potassium phosphate over 4 min. The column eluate was monitored at 294 nm. Retention times of guanine nucleotide standards were: 5'-GMP, 5.2 min; GDP, 10.9 min; GTP, 18.8 min.

Fluorescence Measurements—Fluorescence spectra were recorded using a Spex Fluorolog 211 spectrophotometer (double monochrometer for excitation; single monochrometer for emission). Temperature was kept constant at 30 °C. Measurements were performed in the ratio mode to correct for variations in intensity of the xenon lamp using a neutral density filter (optical density of 3) as an attenuator in the reference beam. The optical bandwidths on the excitation and emission monochrometers were 2.25 and 4.5 nm, respectively.

Preparation of GTP-A-RF—Guanine nucleotides were bound to ARF as described above. Samples were chilled on ice, and sodium cholate was added to a final concentration of 1%. Bound and free nucleotides were then separated by the method of Fleming and Ross (15). Sephadex G-25 was swollen and equilibrated in 20 mM Na Hepes (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 2 mM MgCl₂, 100 mM NaCl, and 1% sodium cholate. Polypropylene columns (48 × 8 mm; Isolab, Inc.) were poured with a bed volume of 3 ml. Intestinal solvant was removed by centrifugation at 1000 rpm for 5 min in a Beckman model TJ-6 centrifuge. Samples (100 μl) were then applied to the ion-exchange material washed with 10 ml of 100 mM NaCl, 10 mM MgCl₂, 0.1% sodium dodecyl sulfate, and 1.0% Triton X-100 (1000 rpm for 5 min). Greater than 80% of protein was routinely recovered in a volume of 140–175 μl. Less than 0.03% of the unbound nucleotide was recovered in the voided fraction.

Miscellaneous—Cyc- membranes were prepared according to Ross and Gilman (16). Protein concentrations were determined by the method of Schaffer and Weiseman (17). ³²P-labeled nucleotides were synthesized as previously described (18). [³²S]GTPγS was purchased from ICN Biochemicals, Inc.

RESULTS

Purification of ARF—Purification of ARF from bovine brain membranes was achieved with essentially the same four-step procedure described for rabbit liver (8). Starting with 15 g of brain membranes, one can obtain 2–5 mg of pure ARF with an overall recovery of 10% (Table I). This is about 10 times the yield from a comparable rabbit liver preparation; this results from the greater abundance of ARF in brain and the greater stability of the protein during purification. Only 300-fold purification was necessary to obtain pure ARF from brain membranes, compared to the 1800-fold purification required previously. Like ARF from rabbit liver, the purified material from bovine brain appears as a doublet (Mr ~ 21,000) in sodium dodecyl sulfate-polyacrylamide gels. Partial resolution of these two forms of ARF revealed very similar specific activities. The addition of a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, N-tosyl-L-phenylalanine chloromethyl ketone, and bacitracin) appeared to increase the relative amount of the larger band; however, the relationship of these bands remains open to question.

The presence of AMF was required for recovery of any ARF activity from the DEAE-Sephadex column using rabbit liver as the source of membranes (8). A single peak of ARF activity was observed when AMF or Mg²⁺ alone was included during the chromatography of brain ARF on DEAE-Sephalchel (Fig. 1A). Omission of Mg²⁺ or AMF caused ARF to elute in two peaks; the latter was coincidental with G₁ (Fig. 1B).

The lability of ARF from rabbit liver during chromatography on heptanoyl-Sepharose made it necessary to elute activity with a step gradient (8). This was not the case with ARF from brain, and a linear gradient of sodium cholate resulted in elution of ARF that was between 20 and 60% pure. The predominant contaminants after such chromatography are the a subunit of G₃ and an unknown protein (Mr = 26,000). The inclusion of AMF or MgCl₂ during chromatography on heptanoyl-Sepharose was necessary for the elution of ARF in a single peak. Omission of AMF or MgCl₂ at this step caused extreme broadening of the peak of ARF activity.

Studies on the effects of AMF on the chromatographic behavior of ARF led to the realization that AMF or MgCl₂ alone markedly stabilizes ARF activity in cholate extracts of brain membranes. The half-life of ARF activity in cholate extracts was 2.5 days at 4 °C in the absence of MgCl₂ (and presence of 1 mM EDTA) and 14 days in 5 mM MgCl₂. MnCl₂, but not CaCl₂, could also stabilize ARF activity in such extracts. Results of purification in which all buffers contained 5 mM free MgCl₂ (but no added AMF or F⁺) were the same or better (recoveries) than those done in AMF, suggesting that both the stability and the chromatographic behavior of ARF are dependent on the divalent cation.

Velocity Sedimentation and Purification in the Absence of Detergent—Electrophoretically purified ARF sediments as a mono-disperse species with an S value of 2.10 (identical to ARF from liver; 8) when applied to a linear sucrose gradient
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TABLE I
Purification of ARF from bovine brain membranes

| Step | Volume | Activity | Protein | Specific activity | Recovery |
|------|--------|----------|---------|-----------------|----------|
| 1. Extract | 1,750 | 1,360 | 3.65 | 372 | 100 |
| 2. DEAE-Sephacel | 820 | 2,170 | 1.93 | 1,120 | 74 |
| 3. Ultrogel AcA 44 | 154 | 5,240 | 0.93 | 5,630 | 34 |
| 4. Heptylamine-Sepharose | 153 | 2,900 | 0.11 | 26,400 | 19 |
| 5a. Preparative SDS-PAGE | 37 | 3,800 | 0.08 | 6,300 | 10 |
| 5b. Preparative HPLC | 1.2 | 200,000 | 1.17 | 171,000 | 15 |

Chromatography of ARF on DEAE-Sephacel in the absence (A) and presence (B) of AMF. Cholate extracts of brain membranes were loaded onto a 1.4-liter column of DEAE-Sephacel and eluted with a linear gradient of 0–250 mM NaCl as described under "Materials and Methods." Buffers containing 1% sodium cholate. Recovery of activity is nearly complete. In the absence of added detergent, recovery is poor (8–20%), and activity is found throughout the gradient (Fig. 2). When ARF from the heptylamine-Sepharose column (~30% pure) is applied to sucrose gradients with or without cholate, a single peak of activity is observed and recovery of activity is high (80–90%). However, the activity profile from gradients centrifuged in the absence of added detergent is consistently broader than those that contain cholate. Thus, electrophoresis of ARF in the presence of sodium dodecyl sulfate appears to change its behavior during sucrose density gradient centrifugation in the absence of detergent.

These results suggested that alternative methods of purification should be explored, and it was found that ARF can be purified to homogeneity with good recovery by gel filtration (Bio-Sil TSK) chromatography in the absence of detergents (Fig. 3). The main contaminants in the preparation after chromatography on heptylamine-Sepharose seem to aggregate in the absence of detergent and appear in the voided fractions of the TSK column. ARF elutes at the position expected for a 21,000-dalton protein. The specific activity of ARF (up to 400,000 units/mg) purified in this way is 50–400% greater than comparable samples prepared by gel electrophoresis in the presence of sodium dodecyl sulfate. HPLC purified ARF was used in all subsequent experiments.

Binding of Guanine Nucleotides—ARF binds a number of guanine nucleotides, including GTP, GDP, and GTPyS. Such containing 1% sodium cholate. Recovery of activity is nearly complete. In the absence of added detergent, recovery is poor (8–20%), and activity is found throughout the gradient (Fig. 2).
binding is observed in the presence of lipid, Mg$^{2+}$, and high ionic strength (Fig. 3; Table II). Rapid dissociation of bound nucleotide is observed at 30°C upon removal of Mg$^{2+}$ (not shown). Binding is optimal between pH 8.0 and 8.5. The maximal binding of guanine nucleotides was routinely be-

TABLE II

| Condition          | Relative binding % |
|--------------------|--------------------|
| Complete           | 100                |
| 0.4 M NaCl         | 73                 |
| 0.2 M NaCl         | 39                 |
| 0.1 M NaCl         | 24                 |
| 0 NaCl             | 14                 |
| 0.4 M Na Hepes, pH 8 | 19                |
| 0.4 M potassium phosphate, pH 8 | 74 |
| 1 mM DMPC          | 58                 |
| 0.5 mM DMPC        | 46                 |
| 0.1 mM DMPC        | 26                 |
| 0 DMPC             | 1.5                |
| 0 MgCl$_2$ + 1 mM EDTA | 0.0            |

Log Nucleotide Concentration

![Fig. 4. Scatchard analysis of binding of GTP$\gamma$S to ARF.](image)

![Fig. 5. Competition by nucleotides for the binding of $^{[35]S}$GTP$\gamma$S to ARF. HPLC-purified ARF (5 nM) was incubated with $^{[35]S}$GTP$\gamma$S (10 nM, 10$^6$ cpm/point) and the indicated unlabeled nucleotides for 2 h at 30°C, as described under "Materials and Methods." Duplicate aliquots were taken, and the amount of bound $^{[35]S}$GTP$\gamma$S was determined by filtration assay described under "Materials and Methods." Competing nucleotides used were GTP$\gamma$S (A), GTP (B), GDP (C), GMP (D), ADP (E), and ATP (F). Maximum binding for this preparation of ARF was approximately 0.55 mol of GTP$\gamma$S/mol of ARF.](image)
containing bound GDP, GTP, or GTP"S was examined by excitation at 290 nm. When GTP or GTP"S was bound to ARF, the intrinsic fluorescence was increased by about 110% over that observed with the GDP form of the protein (Fig. 6). The spectra for GTP- and GTP"S-ligated ARF were superimposable, consistent with the failure to observe GTPase activity in the preparation. The addition of AMP had no effect on fluorescence, and the spectrum of ARF in AMF was identical to that of GDP-ARF.

The intimate association between the binding of GTP"S and the increase in intrinsic fluorescence as a function of time is demonstrated in Fig. 7. When GDP was added instead of GTP"S, the intrinsic fluorescence did not change during the 2-h incubation at 30 °C.

Guanine Nucleotide Requirement for ARF Activity—With the findings that ARF binds guanine nucleotides and that conditions required for binding are very similar to those previously described for the expression of ARF activity, it was possible to reexamine the site of action of GTP required for cholera toxin-catalyzed ADP-ribosylation of G. Purified ARF was first incubated with either no guanine nucleotide, GDP, GTP, or GTP"S for 2 h at 30 °C. Cholate (1%) was added at 0 °C to disrupt vesicles, and each sample was then applied to a G-25 column to separate bound and free nucleotides, as described under "Materials and Methods." The various forms of ARF were then assayed for their ability to support cholera toxin-dependent activation of G, either in the presence or absence of added GTP. Carryover of free nucleotides was less than 0.03%, sufficient to bind to less than 1% of the G, in the reaction mixture. ARF that had not been preincubated (not shown) or that had been incubated with no guanine nucleotide or with GDP was unable to support ADP-ribosylation unless GTP was present during the incubation with toxin and G, (Fig. 8). When GTP was added to these samples, there was a lag of up to 5 min before the rate of activation became linear. ARF containing bound GTP or GTP"S was able to support activation of up to 30% of the G, present, even when no GTP was added during the second incubation (Fig. 8). There was no lag with GTP-ARF or GTP"S-ARF. Activation was maximal when GTP was included in the second incubation. The decreased rate and extent of activation seen when samples first incubated with GTP or GTP"S were assayed without GTP in the second incubation results in part from the dissociation of nucleotides from ARF. In addition, ADP-ribosylation of G, is very unstable in the absence of GTP; and the assay utilized depends on the ability of G, to stimulate adenylate cyclase activity. These results demonstrate that binding of GTP to G, is not required for ADP-ribosylation catalyzed by cholera toxin, as previously believed. Rather, the requirement for GTP in this reaction is ascribable to the nucleotide binding site on ARF. The lag noted previously (8) is due to the rate-limiting binding of GTP to ARF.

These results also clarify another ambiguity. We previously reported that G, GTP"S is not a substrate for cholera toxin, yet GTP"S can partially support the toxin-catalyzed modification of G, when added to reaction mixtures containing basal G, (19). It is now clear that, indeed, G, GTP"S is not a substrate for cholera toxin; when GTP"S is added to basal G, and ARF in the presence of cholera toxin, the ADP-ribosylation that occurs is the result of the interaction of ARF-GTP"S with basal G,.

**DISCUSSION**

ARF, a 21,000-dalton membrane-bound protein, is necessary for the ADP-ribosylation of G, by cholera toxin. Evidence
discussed previously suggests that ARF interacts with G, rather than the toxin (8). The data presented above indicate that ARF binds guanine nucleotides with high affinity and, as purified, contains 1 mol of GDP/mol of protein. Although the physiological role of ARF is unknown and effects of ARF on adenylate cyclase activity have not yet been detected, these observations raise the possibility that two guanine nucleotide-binding regulatory proteins are involved in the pathway responsible for stimulation of adenylate cyclase activity.

Binding of GTP (or GTP•P•S) to ARF is necessary and sufficient for the ADP-ribosylation of G,. Nucleotide hydrolysis is not necessary, and GDP-ARF does not support the covalent modification. Although hydrolysis of GTP by ARF has not been detected under the conditions tested, the finding that ARF is purified as a complex with GDP and analogues with other proteins suggest that ARF may in fact be a GTPase. A component necessary for GTPase activity may have been resolved from ARF during purification. Precedent for such comes from bacterial elongation factor Tu, which binds GTP but hydrolyzes the nucleotide only when bound to ribosomes in the presence of mRNA and aminoacyl-tRNA (20). A systematic search to detect an additional hypothetical factor will be necessary. Although G, is an obvious candidate, the rate of GTP hydrolysis observed in the presence of G, was unchanged by the addition of equimolar amounts of GDP, under conditions where ARF binds guanine nucleotides. These negative data serve only to set an upper limit to the rate of any hydrolysis of GTP by ARF under these conditions. This limit (0.015 mm⁻¹) is close to the rate of hydrolysis of GTP by the transforming viral ras proteins (21).

There are similarities between the guanine nucleotide-binding properties of ARF and those of G proteins. These include high affinity and specificity for guanine nucleotides and retention of bound GDP during purification. Differences are also apparent, including the lack of intrinsic GTPase activity of ARF, the lack of effect of All and F(32) and the absolute requirement for Mg⁴⁺ for nucleotide binding to ARF. The failure of ARF to bind guanine nucleotides in the presence of detergent and the requirement for high ionic strength for such binding are also notable. These characteristics, in addition to partial denaturation of ARF during purification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the very limited amount of protein previously available, account for our earlier failure to detect the guanine nucleotide-binding capacity of ARF.

It is more tempting to compare ARF and the ras gene products, although this exercise is clearly compromised by ignorance. ARF and the p21 products of the ras genes are membrane-bound, GTP-binding proteins of similar mass. Both are found in high relative abundances in brain. Of interest, both have been implicated as components of adenylyl cyclase. The yeast ras gene products, which are approximately 40,000-dalton proteins, and the smaller p21 proteins of viral and human origin clearly stimulate yeast adenylyl cyclase activity (22) but cannot replace G, as an activator of adenylyl cyclases of higher organisms (23). It is not known if there is a direct interaction between the ras proteins and adenylyl cyclase or if the GTP binding site on ras proteins accounts for the sensitivity of yeast adenylyl cyclase to guanine nucleotides. In yeast it is possible that ras influences adenylyl cyclase via the intermediary of a distinct G-like protein.

so, the analogy with ARF may become obvious. Unfortunately, our simple attempts to test this relationship have yet to yield positive results. These tests have utilized viral ras proteins expressed as fusion proteins in Escherichia coli and monoclonal antibodies to the viral proteins (24, 25). p21 has no ARF activity. Antibodies to the viral ras proteins do not appear to interact with ARF. Limited amino acid sequence information on ARF reveals no striking homology with ras proteins. More detailed characterization of ARF will be necessary to allow further comparison.

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