Characterization of Recombinant and Endogenous ADP-Ribosylation Factors Synthesized in Sf9 Insect Cells†

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ABSTRACT: ADP-ribosylation factors (ARFs) are a family of highly conserved, 20-kDa guanine nucleotide-binding proteins that participate in protein trafficking and enhance cholera toxin-catalyzed ADP-ribosylation. ARF 2 from bovine retinal cDNA was expressed in Sf9 insect cells using recombinant baculovirus and compared to the major insect cell ARF (Sf9 ARF) and to recombinant ARF 2 expressed in *Escherichia coli* (*E. coli* rARF 2). The 150000*g* supernatant and particulate fractions of freeze-thawed, recombinant ARF 2 baculovirus-infected cells contained immunoreactive proteins of 20 and 21 kDa at significantly higher levels than were found in uninfected cells. Infected Sf9 cells incorporated [^{3}H]myristate only into the 20-kDa protein. Sf9 cell recombinant ARF 2 (Sf9 rARF 2) and Sf9 ARF were separated by isoelectric focusing or ion-exchange chromatography and identified by microsequencing of HPLC-purified tryptic peptides. Sf9 ARF displayed considerable sequence identity to mammalian class I ARFs. Both Sf9 ARF and Sf9 rARF 2 stimulated in a GTP-dependent manner cholera toxin-catalyzed ADP-ribosylation. The K_a for GTP of Sf9 ARF was, however, significantly lower than that of Sf9 rARF 2 or *E. coli* rARF 2. Myristoylation did not significantly affect the ability of ARF 2 to enhance cholera toxin-catalyzed ADP-ribosylation or the K_a for GTP. Despite the sequence identities and the fact that both were synthesized in insect cells, the endogenous Sf9 ARF was functionally different from Sf9 rARF 2.

ADP-Ribosylation factors (ARFs),[1] 20-kDa guanine nucleotide-binding proteins, were originally purified from rabbit liver and bovine brain membranes as cofactors in the cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$, the α subunit of the stimulatory guanine nucleotide-binding protein of the adenylyl cyclase system (Kahn & Gilman, 1984, 1986; Bobak et al., 1990b). It was later found that ARFs isolated from bovine brain membranes and cytosol stimulated both $G_{s\alpha}$-dependent and $G_{s\alpha}$-independent ADP-ribosyltransferase activities of cholera toxin (e.g., ADP-ribosylation of simple guanidino compounds), consistent with ARF being an allosteric activator of the toxin catalytic unit (Tsai et al., 1987, 1988). In all these reactions, ARFs are dependent on GTP or nonhydrolyzable GTP analogues for activity. In mammalian cells, ARFs are believed to function in the protein trafficking pathway in association with other vesicular transport proteins such as β-COP (Donaldson et al., 1991; Serafini et al., 1991), and they may have a role in the formation of nuclear vesicles during mitosis (Boman et al., 1992).

ARFs are ubiquitous, highly conserved proteins that have been identified immunologically in both vertebrate and invertebrate cells (Kahn et al., 1988; Tsai et al., 1991). cDNAs for ARF proteins have been isolated from a variety of eukaryotic species, from *Giardia* to mammals (Price et al., 1988; Sewell & Kahn, 1988; Bobak et al., 1989; Monaco et al., 1990; Tsuchiya et al., 1991; Murtagh et al., 1992). In mammalian species, they encode a family of highly conserved proteins containing 175–181 amino acids (Price et al., 1988; Sewell & Kahn, 1988; Bobak et al., 1989; Monaco et al., 1990; Tsuchiya et al., 1991). Mammalian ARF proteins include consensus sequences believed to be responsible for guanine nucleotide binding and GTP hydrolysis (Price et al., 1990). On the basis of size, phylogenetic analysis, and amino acid identity, the mammalian ARFs fall into three classes (I, II, and III) (Tsuchiya et al., 1991); members of each class can stimulate cholera toxin-catalyzed reactions (Weiss et al., 1989; Price et al., 1992).

An ARF isolated from bovine brain membranes was myristoylated at the amino terminus (Kahn et al., 1988). N-Terminal myristoylation in animal cells has been associated with targeting of proteins to the cytoplasmic side of plasma membranes (Pellman et al., 1985), and with protein–protein interactions in viral coats (Chow et al., 1987). Myristoylated proteins may also be cytoplasmic, e.g., the catalytic unit of cAMP-dependent protein kinase (Carr et al., 1982). In mitochondria, myristoylation can be reversible, leading to the speculation that it may have a regulatory function (Stucki et al., 1989). Nonmyristoylated *Escherichia coli*-synthesized recombinant ARF stimulated cholera toxin activity (Weiss et al., 1989; Price et al., 1992). Nonmyristoylated ARF may not, however, function in protein trafficking (Rothman & Orci, 1992).

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[1] Abbreviations: ARF, ADP-ribosylation factor; rARF 2, recombinant ARF derived from bovine retina ARF 2; Sf9 rARF 2, recombinant ARF 2 expressed in Sf9 cells; *E. coli* rARF 2, recombinant ARF 2 expressed in *E. coli*; sARF I and sARF II, soluble forms of bovine brain ARF; Sf9 ARF, ARF endogenous to Sf9 cells; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; Sf9, *Spodoptera frugiperda* clone 9; DMPC, dimyristoylphosphatidylcholine; DTT, dithiothreitol; CTA, cholera toxin A subunit.

The baculovirus/Sf9 insect cell expression system utilizes the very strong polyhedrin promoter of the baculovirus *Autographa californica* to synthesize high levels of intracellular or exported proteins (Doerfler, 1986; Luckow & Summers, 1988a,b; Miller, 1988). Being eukaryotic, the insect cells are capable of many co- and post-translational protein modifications including myristoylation. In an attempt to understand the effect of myristoylation on the ability of ARF to stimulate cholera toxin-catalyzed ADP-ribosylation, a retinal ARF cDNA (ARF 2) (Price et al., 1988) was expressed in insect cells (Sf9) using a recombinant baculovirus. The activity of the Sf9 recombinant ARF protein was compared to that of the unmyristoylated protein product of the same ARF 2 cDNA expressed in *E. coli*. In the course of the study, an endogenous insect ARF was identified, separated chromatographically from the recombinant Sf9 ARF 2, and partially sequenced. The ability of each of the ARFs to activate cholera toxin was examined.

EXPERIMENTAL PROCEDURES

Cell Culture. The continuous cell line *Spodoptera frugiperda* (Sf9), from the American Type Culture collection, was grown in monolayer culture at room temperature in room air in Grace's insect culture medium supplemented with 10% heat-inactivated fetal bovine serum, fungizone (2.5 μg/mL), gentamicin sulfate (50 μg/mL), lactalbumin hydrolysate (3.33 mg/mL), and Yeastolate (3.33 mg/mL, formula 0860050AJ; Gibco Laboratories, Gaithersburg, MD).

Construction of Recombinant Transfer Vectors. A 1.5-kb cDNA initially isolated from a bovine retinal library (Price et al., 1988) containing 7 bp of 5′-untranslated region, the coding region for ARF 2 (543 bp), and approximately 1000 bp of 3′-untranslated region was cloned into baculovirus vector pVL941 (gift from Dr. Max D. Summers). The 1.5-kb ARF insert was excised from Bluescript KS (+) phagemid vector (Stratagene, La Jolla, CA) with *Eco*RI (Life Technologies Inc., Gaithersburg, MD) and isolated from a low melting point agarose gel by heating and phenol extraction. After reaction with Klenow fragment (Life Technologies Inc.), it was ligated into *Bam*HI-linearized, Klenow-treated vector pVL941. Competent HB101 *E. coli* (Life Technologies Inc.) were transformed with the ligated construct according to supplier specifications. Clones containing one insert, oriented in the appropriate 5′- to 3′-direction within the baculovirus vectors, were selected by restriction analysis and used for transfection.

Bovine $G_{o\alpha}$ cDNA containing the coding region, 60 bp of 5′-untranslated region, and 139 bp of 3′-untranslated region was ligated into pVL941 to serve as a control for expression of foreign proteins in insect cells.

Transfection. Plasmid DNA used for transfection was harvested from $CsCl_2$ gradients prepared from chloramphenicol-amplified *E. coli* clones lysed with alkaline lysozyme and 0.1% Triton X-100 (Sigma, St. Louis, MO). After removal of ethidium bromide (Bio-Rad, Richmond, CA) (Maniatis et al., 1982) and $CsCl_2$, plasmid DNA was precipitated with 2.5 volumes of ethanol. Preparations were treated with RNAse (Sigma), phenol-extracted, ethanol-precipitated after adjustment to 0.3 M NaCl, and washed with 70% ethanol prior to transfection.

Baculovirus genomic DNA was isolated from extracellular virus recovered from the medium of Sf9 cells infected with wild-type virus according to a standard protocol (Summers & Smith, 1987), except that sucrose gradient centrifugation was omitted. The genomic DNA was stored at 4 °C in sterile 1 mM Tris-HCl (pH 8.0)/0.1 mM EDTA.

Adherent Sf9 cells in 25 cm^2 Costar flasks (3×10^6 cells/flask) were transfected with plasmid DNA (1–10 μg) and wild-type genomic baculovirus DNA (0.5 μg) by calcium phosphate precipitation. In 5–10 days, when baculovirus polyhedron inclusion bodies were present in the majority of cells, the medium was recovered, centrifuged to remove cellular debris, and stored under sterile conditions at 4 °C. This medium was later screened in a plaque assay to detect recombinant baculovirus containing ARF cDNA without the wild-type polyhedrin gene.

Plaque Assay for Isolation of Recombinant Virus. Recombinant baculovirus containing ARF cDNA was isolated using a plaque assay as described by Summers and Smith (1987). The 1.5-kb cDNA was used to screen recombinant virus. Routinely, five to seven rounds of screening and purification were performed to reach the point where, with amplification of the recombinant baculovirus by sequential infections, ARF protein was produced without the presence of wild-type baculovirus polyhedron inclusions. Recombinant virus was stored in Sf9 cell medium at 4 °C.

Purification of Endogenous ARF from Sf9 Cells. Uninfected cells (5 L) were grown in suspension culture to a density of 1×10^6 cells/mL and harvested. The cell pellets were lysed by repeated freezing and thawing. Cell lysate was then centrifuged (150000g, 2 h). Seven milliliters of the supernatant (142 mg of protein) was applied to a column (2.5 × 90 cm) of Ultrogel AcA 54 that was equilibrated and eluted with buffer A [20 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mM DTT/1 mM benzamidine/1 mM NaN_3/5 mM $MgCl_2$/0.25 M sucrose] containing 0.1 M NaCl. Active fractions were combined (28 mL, 2.7 mg of protein), diluted with 28 mL of buffer A, and applied to a column (1 × 39 cm) of hydroxylapatite equilibrated with buffer A. Proteins were eluted with a linear gradient of potassium phosphate, pH 7.5 (0 – 60 mM; 100 mL/100 mL), in buffer A (Figure 1A).

Purification of Recombinant ARF 2 from Baculovirus-Infected Sf9 Cells. Medium recovered from infected Sf9 cells was used as a source of recombinant baculovirus stock after passage through a 0.22-μm filter. Sf9 cells (1×10^6 cells/mL) plated on 25 cm^2 flasks were infected in duplicate with serial dilutions of the viral stock. The cells were harvested after 72 h and analyzed for rARF 2 protein via immunoblotting. The dilution that yielded maximal protein production was determined for each viral stock.

Sf9 cells (16 mL, 5×10^9 cells) infected with recombinant baculovirus were frozen and thawed 4 times and centrifuged (150000*g*, 2 h, at 4 °C). A sample (12.5 mL, 95 mg of protein) of the supernatant (total volume 100 mL) was applied to a column (2.5 × 90 cm) of Ultrogel AcA 54 equilibrated and eluted with buffer A containing 0.1 M NaCl. Active fractions (84 mL, 18.2 mg of protein; see Figure 1) were diluted with 84 mL of buffer A and applied to a column (1 × 39 cm) of hydroxylapatite equilibrated with buffer A. Proteins were eluted as described for purification of endogenous ARF (Figure 1B).

Sequencing of Sf9 ARF and Sf9 rARF 2. Protein sequencing and mass spectroscopy were performed by Dr. William Lane of Harvard Microchem on HPLC-purified tryptic peptides. Trypsin digestion of total protein was performed on nitrocellulose following identification of the protein band by Ponceau Red staining.

Quantification and Characterization of Sf9 rARF. Sf9 rARF was quantified by densitometric scanning (laser

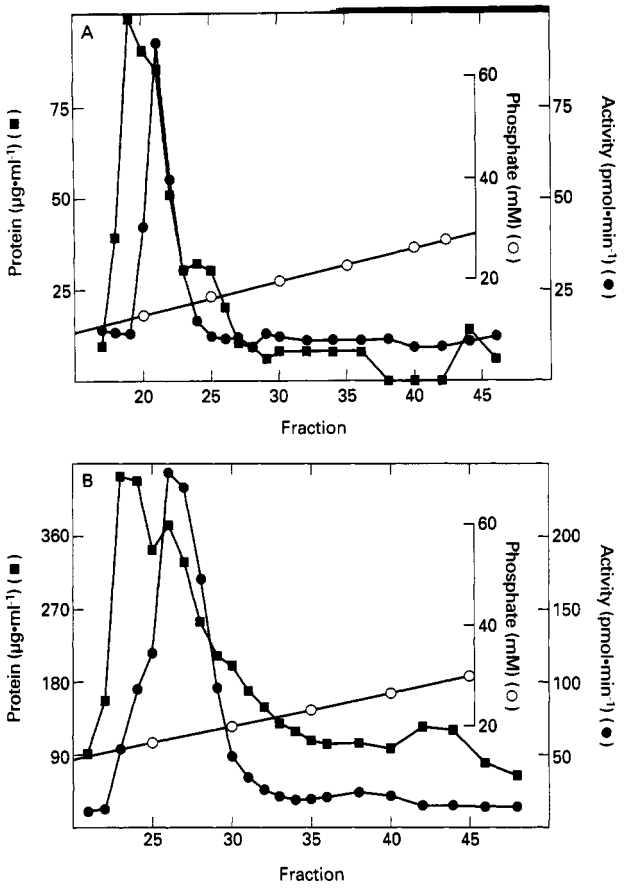

FIGURE 1: Purification of Sf9 ARF and Sf9 rARF 2 by hydroxylapatite chromatography. (A) Ultrogel AcA 54-purified Sf9 ARF (2.7 mg) was applied to a column (1.0 × 39 cm) of hydroxylapatite equilibrated with buffer A [20 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mM DTT/1 mM benzamidine/1 mM NaN_3/5 mM $MgCl_2$/0.25 M sucrose] containing 0.1 M NaCl. Proteins (■) were eluted with a linear gradient of potassium phosphate [0–60 mM in buffer A, 100 mL/100 mL (○)]. Fractions (3 mL) were assayed for ARF activity (●) under standard conditions. (B) Ultrogel AcA 54-purified Sf9 rARF 2 (18.2 mg) was applied to hydroxylapatite, eluted, and assayed as described in (A).

densitometer; Pharmacia LKB Biotechnology, Piscataway, NJ) of immunoblots. Three concentrations of recombinant ARF in 100000*g* supernatant or particulate fractions were subjected to electrophoresis in an SDS–polyacrylamide gel (Laemmli, 1970) and transferred to Immobilon-P (Millipore, Bedford, MA). Blocked membranes were incubated overnight with anti-bovine sARF II rabbit IgG (Tsai et al., 1991), and for 2 h with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega, Madison, WI). Bovine brain sARF II was used as a standard. Blots with acylated proteins were sprayed with En^{3}Hance (NEN, Boston, MA) before autoradiography.

Isoelectric Focusing. Protein samples (1–3 μg) were applied, 2 cm from the cathode, to Pharmacia—LKB Ampholine PAG plates (pH 3.5–9.5) and run for 3000–4000 Vh (3000 V, 150 mA). Isoelectric points were determined on the basis of Bio-Rad prestained and Pharmacia standards. Isoelectric focusing separated Sf9 ARF (p*I* ~4.9, ~20 kDa) from the two recombinant immunoreactive proteins in the 150000*g* supernatant. The p*I* was ~5.2 for the unmyristoylated ~21-kDa ARF and 6.0 for the myristoylated ~20 kDa ARF. Isoelectric focusing was a sensitive way to detect cross-contamination of endogenous and recombinant ARFs.

ARF Activity. ARF stimulation of cholera toxin activity was determined in an NAD:agmatine ADP-ribosyltransferase assay. Unless otherwise noted, reaction mixtures contained 50 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 30 μg of ovalbumin, 20 mM DTT, 100 μM nicotinamide [U^{14}C]-adenine dinucleotide (50 000 cpm), 10 mM agmatine, 100 μM GTP, 1 μg of CTA (List Biological Laboratories, Campbell, CA) and sample as indicated (total volume, 0.3 mL). After incubation for 60 min at 30 °C, two 100-μL samples were applied to AG1-X2 columns (0.5 × 4 cm) followed by four washes with 1.25 mL of H_2O (Noda et al., 1989). Eluates were collected for radioassay of [^{14}C]ADP-ribosylagmatine. All assays were run in duplicate.

Acylation. For labeling with [9,10-^{3}H]myristate (39 Ci/mmol, NEN) or [9,10-^{3}H]palmitate (30 Ci/mmol, NEN), cells were seeded in 24-well plates (5 × 10^5 cells/well), infected, and grown for 3 days. On day 3, 10 μCi of myristate or palmitate was added, and cells were harvested 4 h later.

Analysis of Acylated ARF. Fatty acids in soluble bovine brain sARF I and sARF II were analyzed as described by Hall and Self (1986). Protein concentration was estimated from Coomassie blue staining of SDS–polyacrylamide gels and Bio-Rad protein assays instead of using amino acid analysis (Schultz et al., 1987).

To quantify [^{3}H]myristate linked to ARF, ^{3}H-myristoylated ARF expressed in Sf9 cells was subjected to electrophoresis in an SDS–polyacrylamide gel and transferred to Immobilon-P. One section of the membrane was incubated with anti-ARF antibodies. Regions of lanes not exposed to antibody that corresponded to immunoreactive material were cut from the membrane and washed with chloroform/methanol (2:1, v/v) to remove noncovalently bound fatty acids. Acid methanolysis of the proteins was performed by heating the Immobilon-P (48 h, 110 °C) in 83% methanol/2 M HCl, containing 0.1 mg each of myristic acid and palmitic acid (Sigma). Lipids were then extracted with petroleum ether (1 mL). After addition of 0.1 mg each of methyl myristate and methyl palmitate, samples were dried under a stream of nitrogen, suspended in methanol, and run on a reversed-phase C_{18} HPLC column (Supelco, Inc., Belleforte, PA) (4.6 mm × 30 cm). The mobile phase was 80% acetonitrile in H_2O, flow rate 1 mL/min. Lipids and esters were detected at 220 nm. Fractions (1 mL) were collected for radioassay in a liquid scintillation counter. Elution profiles of radioactivity and absorbance of the standards were compared.

Protein Purification. sARF II was purified from bovine brain supernatant (Tsai et al., 1988). rARF 2, synthesized in *E. coli*, was purified as described by Price et al. (1992).

RESULTS

Quantification of Sf9 rARF 2 Protein. Sf9 cells infected with baculovirus containing pVL941-ARF 1.5 produced DNA and mRNA that hybridized to an ARF cDNA (data not shown). All Sf9 cells, uninfected or those infected for 3 days with recombinant baculovirus, contained an immunoreactive protein of 20 kDa that reacted with anti-bovine sARF II polyclonal antibodies (data not shown). Densitometry of immunoblots showed that the density of staining was linear with the amount of ARF in the range of 0.1–0.5 μg per lane (data not shown). Uninfected cells typically contained about 2 μg of ARF/mg of protein in the 100000*g* supernatant and 1.6 μg of ARF/mg of protein in the membrane fraction, which contained ~30% of total cellular ARF (Table I). The total amount of Sf9 ARF was reduced to ~1.4 μg/mg of cell protein after infection with the wild-type baculovirus.

Sf9 ARF, in the supernatant or purified through the hydroxylapatite step, was separated by SDS–PAGE into a major immunoreactive 20-kDa band and a minor immunore-

Table I: Amounts of ARF in Uninfected and Infected Sf9 Cells[a]

infection	ARF protein (μg/mg of total protein)		% of total ARF in membrane
	supernatant	membrane	
none	2.0 ± 0.2 (5)	1.6 ± 0.1 (3)	30
wt baculovirus	1.5 ± 0.7 (4)	1.1 ± 0.4 (2)	38
pVL941-G_o	1.6 ± 0.6 (5)	1.0 ± 0.4 (3)	30
pVL941-ARF 1.5	10.1 ± 0.5 (4)	5.0 ± 1.9 (3)	23

[a] Data are mean ± SEM for the number of experiments indicated in parentheses.

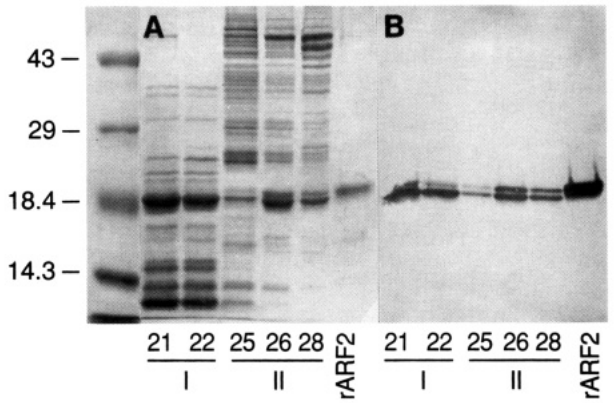

FIGURE 2: Characterization of hydroxylapatite-purified Sf9 rARF 2 and endogenous Sf9 ARF. *E. coli* rARF 2 (5 μg) and 20-μg (A) and 5-μg (B) samples of the indicated hydroxylapatite column fractions (21–28) containing Sf9 ARF (I) and Sf9 rARF 2 (II) were subjected to electrophoresis in 16% polyacrylamide gels with SDS. (A) Coomassie blue-stained gel. (B) Immunoblot: the blocked membranes were incubated with anti-bovine sARF II rabbit IgG overnight and for 2 h with a 1:2000 dilution of anti-rabbit IgG HRP conjugate. Color was developed for 15 min at 4 °C by addition of 50 mg of 4-chloro-1-naphthol dissolved in 20 mL of cold methanol, 80 mL of TBS, and 40 μL of H_2O_2.

active 21-kDa band (Figure 2 and data not shown). Both 20- and 21-kDa immunoreactive proteins were present on immunoblots of Sf9 cells infected with recombinant baculovirus containing ARF cDNA. The 21-kDa protein was a major fraction of total immunoreactive ARF, in contrast to the situation in uninfected Sf9 cells. It was present whether early or late passages of virus were used for infection and represented, as shown by amino-terminal sequencing, ARF that was not myristoylated at the amino terminus. In cells infected with baculovirus containing the pVL941-ARF 1.5 vector, ARF content was increased in both the 100000*g* supernatant and membrane fractions (data not shown). Typically, total ARF was ~9 μg/mg of protein, and as much as 5% of the 100000*g* supernatant protein was ARF. Unmyristoylated ARF in these cells was about 50% of the total in both the 100000*g* supernatant and membrane fractions. Washing with 100 mM NaCl did not release either unmyristoylated or myristoylated protein from the membranes.

Co- or Posttranslational Modification. Membrane ARF is myristoylated on the N-terminal glycine (Kahn et al., 1988). Fatty acid analysis of the two soluble forms of ARF purified from bovine brain in this laboratory (sARF I, sARF II) demonstrated the presence of 0.5–1 mol of myristate per mole of both soluble forms of ARF (Schultz et al., unpublished data). Autoradiograms of SDS–polyacrylamide gels of proteins from Sf9 cells grown in the presence of [^{3}H]palmitic acid or [^{3}H]myristic acid indicated that palmitoylated proteins were present almost exclusively in membranes (data not shown). None of the palmitoylated proteins was ARF. In uninfected cells, the most prominent myristoylated protein was 20 kDa (Figure 3), was present in both supernatant and

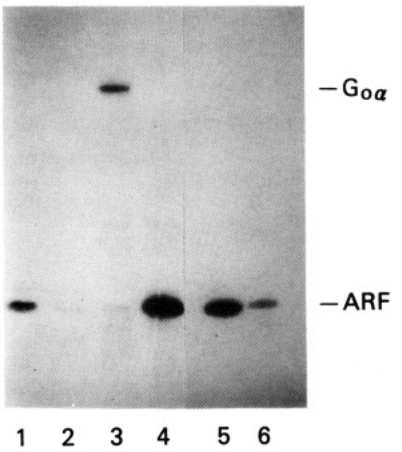

FIGURE 3: ^{3}H-Myristoylation of Sf9 rARF 2. Sf9 cells were infected with pVL941-ARF 1.5 and grown for 3 days. On day 3, [^{3}H]myristate was added to the medium, and cells were harvested after 4 h. In lanes 5 and 6, proteins (75 μg) were separated by electrophoresis in SDS–polyacrylamide gels and transferred to Immobilon-P membranes. Lanes 1–4: Samples of whole homogenate (100 μg of protein), 5-day exposure. Lane 1, uninfected; lane 2, infected with wild-type baculovirus; lane 3, infected with pVL941-$G_{o\alpha}$ virus; lane 4, infected with pVL941-ARF 1.5 virus; lanes 5 and 6, infected with pVL941-ARF 1.5 virus; lane 5, supernatant; lane 6, membrane proteins. Positions of bovine brain $G_{o\alpha}$ and sARF II are indicated on the right.

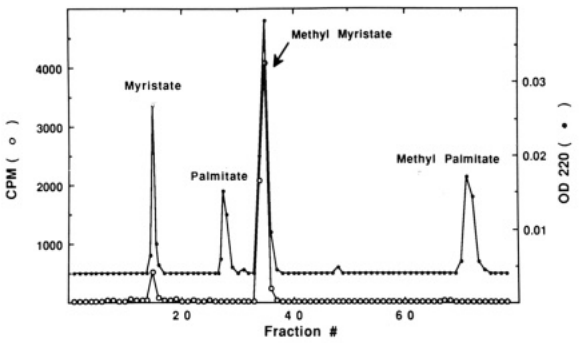

FIGURE 4: High-pressure liquid chromatographic analysis of acid methanolysis products. Acid methanolysis products of Sf9 recombinant ARF 2 were analyzed as described under Experimental Procedures.

membrane fractions (Figure 3, lanes 5 and 6), and was probably Sf9 ARF. In Sf9 cells infected with baculovirus or baculovirus containing a $G_{o\alpha}$ insert, synthesis of ^{3}H-myristoylated ARF was decreased (Figure 3). Substantially larger amounts of myristoylated 20-kDa protein were found in cells infected with pVL941-ARF 1.5. The 20-kDa rARF 2 in the supernatant, as well as that associated with membranes, was myristoylated (Figure 3). The unmyristoylated 21-kDa immunoreactive protein was not blocked to Edman degradation, and the first 15 amino acids (M-G-N-V-F-E-K-L-F-K-S-L-F-G-K-) match the deduced amino acid sequence of bovine ARF 2. HPLC of the acid methanolysis products of ^{3}H-myristoylated ARF confirmed that the fatty acid incorporated into ARF was, in fact, myristic acid (Figure 4). The only products of the reaction were myristic acid and methylmyristic acid. The [^{3}H]myristoyl moiety linked to ARF during incubation of Sf9 cells for 3 h followed by incubation with unlabeled myristate was stable for at least 20 h (data not shown), consistent with a long half-life for the myristoyl moiety.

Purification and Characterization of Sf9 ARF and Sf9 rARF 2. To examine the properties of Sf9 cell ARF, the protein was purified from uninfected cells as described under Experimental Procedures. Sf9 ARF eluted at lower phosphate concentrations from hydroxylapatite than did Sf9 rARF 2

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                10        20        30        40        50        60
bARF 2    MGNVFEKLFKSLFGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIGFNVETVEYKNI
Sf9 ARF             SLFGK     ILMVGLDAAGKTTILYK LGEIVTTIPTIGFNVETVEYKNI
rARF 2    MGNVFEKLFKSLFGK                                          NI

                70        80        90        100       110       120
bARF 2    SFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERVNEAREELTRMLAEDELRDAVLL
Sf9 ARF   SFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRER           MLSEDELR
rARF 2    SFTVWDVG          HYFQN QGLI                     MLAEDELR

                130       140       150       160       170       180
bARF 2    VFVNKQDLPNAMNAAEITDKLGLHSLRQRNWYIQATCATSGDGLYEGLDWLSNQLKNQK
Sf9 ARF         QDLPNAMNAAEITDKLGLHSLR NWYIQ
rARF 2
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FIGURE 5: Comparison of the amino acid sequences of Sf9 ARF and Sf9 rARF 2 and the deduced amino acid sequences of bovine ARF 2. As noted in the text, the amino terminus of nonmyristoylated Sf9 rARF 2 was sequenced directly. Sequences of HPLC-purified tryptic peptides from endogenous Sf9 ARF and Sf9 rARF 2 were also determined. Peptides were either sequenced directly (no underline), or the sequence was determined to be identical to that of bovine ARF 2 (bARF 2) by mass spectroscopy (underline). The only amino acid difference between Sf9 rARF 2 and bARF 2 in the peptides that were analyzed is residue 112 (boldface).

Table II: Effect of ARFs on the NAD:Agmatine ADP-Ribosyltransferase Activity of Cholera Toxin[a]

source of ARF	[GTP] (μM)	ADP-ribosyltransferase act. (pmol·min^{-1}) control	DMPC/cholate	SDS
E. coli rARF 2	0	12.3	18.3	8.8
	0.1	14.3	19.5	8.3
	30	11.5	112.0	11.1
Sf9 rARF 2	0	11.0	10.5	6.7
	0.1	10.0	14.9	6.7
	30	12.0	155.0	6.7
Sf9 ARF	0	8.4	14.7	7.6
	0.1	8.4	58.9	8.5
	30	14.1	52.9	55.8

[a] *E. coli* rARF 2 (2.9 μg), Sf9 rARF 2 (3.8 μg), and Sf9 endogenous ARF (2.5 μg) were added as indicated to assays containing 50 mM potassium phosphate (pH 7.4)/10 mM $MgCl_2$/30 μg of ovalbumin/20 mM DTT/100 μM [*adenine*-U-^{14}C]NAD (50 000 cpm)/10 mM agmatine/1 μg of CTA with GTP, 3 mM DMPC/0.2% cholate, and/or 0.003% SDS as indicated, in a total volume of 0.3 mL. Data are the average of values from duplicate assays, with duplicate samples for radioassay from each. The experiment was replicated twice with similar results.

(Figure 1A,B). Since the amino terminus of Sf9 ARF was blocked, identity was established by microsequencing of tryptic peptides (Figure 5). The Sf9 ARF and Sf9 rARF 2 were identified by immunoreactivity with anti-sARF II antibodies and protein staining of fractions from the hydroxylapatite columns (Figures 1A,B and 2). The major 20-kDa bands were transferred to nitrocellulose, and HPLC-purified tryptic peptides were sequenced (Figure 5). As expected, amino-terminal sequences and sequences of HPLC-purified tryptic peptides derived from Sf9 rARF 2 were identical to corresponding regions in the deduced amino acid sequence of bovine ARF 2 (Figure 5). Sequences and mass spectroscopic analysis of tryptic peptides derived from Sf9 ARF purified from uninfected cells revealed only one amino acid difference from the expected sequence of bovine ARF 2, a class I ARF (Figure 5).

Stimulation of Cholera Toxin-Catalyzed ADP-Ribosylation. Both Sf9 ARF and Sf9 rARF 2 stimulated in a GTP-dependent fashion the cholera toxin-catalyzed ADP-ribosylation of agmatine in the presence of DMPC/cholate (Table II). The endogenous Sf9 ARF, in contrast to Sf9 rARF and *E. coli* rARF 2, was active in the presence of SDS. Further, it exhibited a significantly lower K_a for GTP than did Sf9 rARF 2 or the nonmyristoylated *E. coli* rARF 2 (Figure 6). Sf9 ARF was similar to a soluble ARF from bovine brain (sARF II) in its relatively higher affinity for GTP in DMPC/cholate (Bobak et al., 1990a). Sf9 ARF was distinguished also from both Sf9 and *E. coli* rARF 2 by its relative phospholipid dependence at submicromolar concentrations of GTP (Table II).

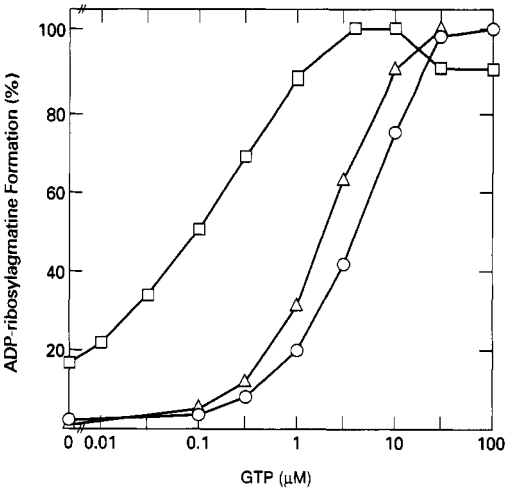

FIGURE 6: Effect of GTP concentration on cholera toxin-stimulating activity of rARF 2 synthesized in *E. coli* or Sf9 cells and Sf9 ARF. Sf9 rARF 2 (3.8 μg) (○), Sf9 endogenous ARF (2.6 μg) (□), and *E. coli* rARF 2 (2.9 μg) (△) were assayed under standard conditions as noted under Experimental Procedures except for the indicated concentrations of GTP and the presence of 3 mM DMPC/0.2% cholate. Maximal ADP-ribosylation in the presence of Sf9 ARF was 47.1 pmol·min^{-1}; that for Sf9 rARF 2, 158 pmol·min^{-1}; and for *E. coli* rARF 2, 118 pmol·min^{-1}.

DISCUSSION

In the studies reported here, we compared the activities of an insect cell ARF (Sf9 ARF) and a recombinant bovine retinal ARF (rARF 2), synthesized in Sf9 cells and in *E. coli*. Endogenous Sf9 ARF, which was present in much smaller amounts than the recombinant protein, was separated from Sf9 rARF 2 by chromatography on hydroxylapatite. The partial amino acid sequence of Sf9 ARF is very similar to that of bovine ARF 2 and other mammalian class I ARFs (Price et al., 1988; Sewell & Kahn, 1988; Bobak et al., 1989). There may be other ARFs in Sf9 cells that are similar to mammalian ARFs of classes II and III (Tsuchiya et al., 1991). The functional roles of the multiple mammalian ARFs remain to be defined.

Endogenous Sf9 ARF differed from rARF 2 synthesized in Sf9 cells or in *E. coli* by its sensitivity to DMPC/cholate and GTP in stimulating cholera toxin ADP-ribosyltransferase activity. Similar to sARF II from bovine brain, Sf9 ARF in the presence of phospholipids activated cholera toxin at submicromolar concentrations of GTP (Bobak et al., 1990a). Both rARF 2 species required substantially higher GTP concentrations. The effects of the recombinant ARFs on the NAD:agmatine ADP-ribosyltransferase activity of cholera toxin were similar with respect to activity per unit of protein, activation by lipid, and GTP concentration dependence. Sf9 rARF 2 contained, however, roughly equal amounts of myristoylated and unmyristoylated ARF, whereas *E. coli* rARF was totally unmyristoylated. The data are thus consistent with the conclusion that myristoylation of rARF has a minimal effect, if any, on its ability to enhance cholera toxin-catalyzed ADP-ribosylation.

Expression of ARF in the insect cells was less than expected compared to expression of the polyhedrin protein. Late in infection with wild-type baculovirus, up to 50% of the protein may be polyhedrin protein (Miller, 1988). Since the level of ^{3}H-myristoylated ARF did not decrease for 20 h following

removal of the labeled fatty acid, rapid degradation of newly synthesized recombinant ARF appears not to have occurred.

It is unclear why both myristoylated ARF and unmyristoylated ARF are found in infected Sf9 cells. Given similar "partial myristoylation" of $G_{o\alpha}$ expressed in Sf9 cells (Graber et al., 1992), the presence of myristoylated and unmyristoylated ARF may reflect a relative limitation in the myristoylation process inherent in the baculovirus/Sf9 cell expression system. Recombinant proteins in baculovirus-infected Sf9 cells are expressed late in infection at a time when host cell intrinsic protein synthesis has stopped. Enzymes like methionine aminopeptidase and *N*-myristoyltransferase are required for removal of the amino-terminal methionine and acylation of the protein, respectively (Gordon, 1990). The low rate of Sf9 protein synthesis that occurs late in baculovirus infection may limit the amount of either of these enzymes, resulting in accumulation of unmyristoylated rARF.

Other explanations for the presence of myristoylated and unmyristoylated rARF in Sf9 cells may lie in the specificity of the insect methylation machinery. *N*-Myristoyltransferase exhibits restricted substrate specificity both in its use of myristoyl-CoA as fatty acyl donor and in the sequence of the amino terminus of the acceptor protein (Towler et al., 1988; Gordon, 1990). The N-terminal sequence of bovine retinal ARF, G-N-V-F-E-K-L-F, with glutamic acid in position 5, differs from the structure favored for N-myristoylation based on studies of peptide substrates (Towler et al., 1988; Gordon, 1990). Depending on the substrate specificity of the insect *N*-myristoyltransferase, this could result in relatively inefficient myristoylation of rARF and accumulation of unmyristoylated rARF.

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