ADP-ribosylation factors (ARFs) are ~20-kDa guanine nucleotide-binding proteins that participate in vesicular transport in the Golgi and other intracellular compartments and stimulate cholera toxin ARF-ribosyltransferase activity. Both GTP binding and hydrolysis are necessary for its physiological functions, although purified mammalian ARF lacks detectable GTPase activity. An ARF GTPase-activating protein (GAP) was purified >15,000-fold from rat spleen cytosol using (NH₄)₂SO₄ precipitation and chromatography on Ultrogel AcA 34, DEAE-Sephacel, heparin-Sepharose, hydroxylapatite, and Ultrogel AcA 44. In fractions (~100-kDa proteins) from Ultrogel AcA 44, a major protein band of ~50 kDa on SDS-polyacrylamide gel electrophoresis correlated with GAP activity, consistent with it being a homodimer, thus differing from an ARF GAP purified from rat liver (Makler, V., Cukierman, E., Rotman, M., Admon, A., and Cassel, D. (1995) J. Biol. Chem. 270, 5232–5237). Purified spleen GAP accelerated hydrolysis of GTP bound to recombinant ARF1, ARF3, ARF5, and ARF6; no effect of NH₂-terminal myristoylation was observed. ARF GAP also activated GTP hydrolysis by ARL1, which is 56% identical in amino acid sequence to ARF1, but lacks ARF activity. ARD1 is a 64-kDa guanine nucleotide-binding protein that contains an 18-kDa ARF domain at its carboxyl terminus; the ARF domain lacks the amino-terminal o-helix found in native ARF and hence is similar to the amino-terminal truncated mutant Δ13ARF1. Both the ARF domain of ARD1 and Δ13ARF1 were poor substrates for ARF GAP. The non-ARF1 domain of ARD1 enhanced the GAPase activity of the ARF domain, but not that of the ARF proteins and Δ13ARF1, *i.e.* it lacks the relatively broad substrate specificity exhibited by ARF GAP.

ADP-ribosylation factors (ARFs)³ are 20 kDa guanine nucleotide-binding proteins, which were originally identified and purified on the basis of their ability to increase the ADP-ribosyltransferase activity of cholera toxin (1–3). In cells, they are apparently involved in the regulation of exocytic and endocytic vesicle transport pathways (3, 4), as well as in the activation of phospholipase D (5, 6). ARFs are highly conserved in eukaryotic cells from *Giardia* to humans; at least six ARFs have been identified in mammalian tissues (7–12). ARF also occurs as the 18-kDa carboxyl-terminal domain of a 64-kDa protein termed ARD1 (13). The amino- and carboxyl-terminal domains of ARD1 synthesized in *Escherichia coli*, interact functionally and the NH₂-terminal protein stimulates GAPase activity of the ARF-like domain, *i.e.* serves as its GAP (14). A family of proteins, termed ARLs, for ARF-like proteins, are very similar to ARFs in structure, but lack the ability to activate cholera toxin (15–18).

ARF proteins are active when GTP is bound. The exchange of bound GDP for GTP, which is necessary to form ARF GTP, is accelerated by a guanine nucleotide-exchange protein or GEP. Membrane-associated GEP activity has been described (19, 20), and a GEP from bovine brain cytosol has been partially purified (21). Inactivation results from the hydrolysis of bound GTP, which requires interaction with a GAP or GAPase-activating protein, as ARF itself has no detectable GAPase activity (22–24). Randazzo and Kahn (23) studied an ARF GAP activity in bovine brain membrane extracts that was stimulated by acidic phospholipids. More recently, the purification (24) and cloning (25) of an ARF GAP from rat liver cytosol has been reported. We describe here the purification and characterization of an ARF GAP from rat spleen cytosol that appears to have a rather broad substrate specificity as it activated GAP hydrolysis by mammalian ARFs from all three classes, with and without myristoylation, as well as by ARF-like ARL proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—**[³²P]GTP and [³⁵S]GTP were purchased from Dupont NEN. Recombinant rARF1, rARF5, rARF6, and recombinant myristoylated ARF1 (myrARF1), ARF5 (myrARF5), and ARF6 (myrARF6) were prepared as described (26, 27). Native ARF1 and ARF3 were purified from bovine brain (2, 28). "Mixed ARFs," a fraction prepared by gel filtration of bovine brain cytosol (28), contained primarily ARF1 and ARF3, as determined by Western blot analysis. Δ13ARF1 and ARL1 were purified as described by Hong et al. (29) and Zhang et al. (30), respectively. Preparation of ARD1 and its two domains were described by Mishima et al. (13) and Vitale et al. (14).

**Purification of ARF GAP—**Rat spleen (320 g) was homogenized (Polytron) in 4 volumes (w/v) of TENDSP buffer (20 mM Tris-Cl, pH 8.0, 1 mM EDTA/1 mM Na₃VO₄, 1 mM diithiothreitol, 0.25 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride) with leupeptin, antipain, and soybean and lima bean trypsin inhibitors, each 1 μg/ml. The homogenate was centrifuged (12,000 × g, 60 min), and the resulting supernatant was then centrifuged at 175,000 × g for 75 min (Beckman SW 41 rotor, 37,000 rpm). Solid (NH₄)₂SO₄ was added to the supernatant to 40% saturation (pH maintained ~7.5). After 45 min at 4 °C, precipitated proteins were
Substrate Specificity of ARF GAP and ARD1 GAP Domain

TABLE I

Purification of GAP from rat spleen cytosol

| Purification step | Protein | Specific activity | Purification | Recovery |
|-------------------|---------|------------------|--------------|----------|
|                   | mg      | fold | %        |          |
| Ultratrog AcA 34  | 1500    | 0.125 | 1        | 100      |
| DEAE-Sephadex     | 220     | 0.33  | 2.64     | 40       |
| Heparin-Sepharose | 17      | 1.25  | 10       | 11       |
| Hyaluronic acid   | 0.3     | 7.69  | 81.5     | 1.2      |
| Ultratrog AcA 44  | 0.014   | 111.1 | 889      | 0.8      |

ΔGTP is the difference between the amounts of [α-32P]GTP bound after incubation without and with GAP, i.e. activity is the fractional decrease during the assay in bound GTP due to the presence of GAP. A unit of GAP activity is defined as the amount causing hydrolysis of 50% of GTP bound to 0.24 µg of mixed ARFs in 10 min at 30 °C.

RESULTS AND DISCUSSION

For initial experiments, GAP assay I, based on GAP activation of CTA-catalyzed ADP-ribosyltransferase activity on or in the hydrolysis of GTP bound to GDP, quantified after thin layer chromatography (TLC). To measure GAP activation of CTA (assay II), ARF GTP was prepared by incubating 0.5 µg of mixed ARFs, 30 µg of bovine serum albumin, 0.1 µg of phosphatidylserine, and 5 µM GTP with TENDSP buffer (total volume 40 µl) at 30 °C for 2 h before transfer to an ice bath. To the ARF GTP mixture, the GAP preparation (10 µl) was added and the total volume adjusted to 100 µl with TENDSP buffer. After incubation at 30 °C for 10 min, mixtures were placed in an ice bath after incubation of components of the CTA assay, i.e. 100 µl of a solution containing 60 µg of ovalbumin, 60 µM Cibacron blue, 40 µg of phosphatidylserine, and 2 µg of CTA plus 100 µl of a solution of 150 mM potassium phosphate, pH 7.5, 1.5 mM MgCl2, 50 mM ATP, 60 mM dithiothreitol, 50 mM ammonium acetate, and 0.6 mg of adenine-[14C]NAD (105 cpm/final volume 300 µl). After incubation at 30 °C for 60 min, [14C]ADP-ribosylatugm was isolated for radioassay (2). ARF activity was expressed as the increase in CTA activity (product formed) due to its activation. ARF activity was, of course, decreased by GAP-catalyzed enhancement of GTP hydrolysis.

The PLC-based assay II for ARF GAP was a modification of that described by Randazzo and Kahn (23). ARF (0.6–1.2 µM) was incubated with 0.5 µM [α-32P]GTP in 20 mM Tris-Cl, pH 8.0, 1 mM MgCl2, 0.1% Triton X-100, 2 mM dithiothreitol, and 30 µg of bovine serum albumin (total volume: 60 µl) at 30 °C for 30 min (recombinant ARFs) or 2 h (native ARFs). Samples (10 µl) were then diluted with 40 µl of the same buffer containing 130 µM PIP2, with or without ARF GAP (final volume: 60 µl) and incubated for 10 min at 30 °C before dilution with 2 ml of ice-cold 20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 5 mM MgCl2, and 2 mM dithiothreitol. Protein-bound nucleotides were collected on nitrocellulose paper and eluted in 250 µl of 2 M formic acid. Samples of eluates were applied to polyethyleneimine-cellulose plates (23) and the remainder was used for radioassay to quantify total nucleotide. Plates were developed with 1 M LiCl/1 M formic acid to separate [α-32P]GTP and [α-32P]GDP, which were quantified using a PhosphorImager (Molecular Dynamics). Because PIP2 (used in the GAP assay) causes dissociation of GDP from ARF (28), activity was calculated as ΔGTP/GTPo, where

ΔGTP = GTPo - GTPf

where GTPo and GTPf are the amounts of [α-32P]GTP bound to ARF in the absence and presence of GAP, respectively.
concentrations of PIP_2 and of lesser magnitude than those described for the brain GAP. Effects of PIP_2 on the ARF GAP purified from liver cytosol (24) were also smaller than those reported by Randazzo and Kahn (23). It was notable that the magnitude of the effects of PIP_2 (and of mixed phosphatidylinositols) in the studies of Makler et al. (24) appeared to vary with the amount of GAP in the assay and its purity. Although it is difficult with the information available, to evaluate conclusively the physiological significance of PIP_2 effects on GAP activity, it appears that PIP_2 may well have a role in more than one aspect of ARF function. The notable enhancement of ARF activation of phospholipase D by PIP_2 has been well documented (31, 32).

As reported by Terui et al. (33), PIP_2 appeared to accelerate...
release of GDP from ARF. Dissociation of GDP from ARF3 was faster than from ARF1, as seen, for example, in the inset in Fig. 2. Although the amount of bound \( {^{[35]}S} \text{GTP} \) remained constant during incubation with GAP, the total amount of bound \( {^{[32]}P} \text{GTP} \) plus \( {^{[32]}P} \text{GDP} \) declined as GTP hydrolysis proceeded (Fig. 5), presumably due to dissociation of bound \( {^{[32]}P} \text{GDP} \). As effects of differing assay conditions on bound GTP were not observed, GAP activity was expressed as the fractional decrease in bound GTP.

ARF GAP also enhanced the GTPase activity of the ARF-like ARL1 and mutant ARF1 lacking 13 amino acids at the NH\(_2\)-terminus (Fig. 6), albeit apparently less effectively than it enhanced the GTPase activity of the native or recombinant ARFs. Myristoylation of the NH\(_2\)-terminal deletion mutant Δ13ARF1 (Fig. 6) had no apparent effect on its activity, although the interpretation of the observation is necessarily limited, as it is for the data with the recombinant intact ARF proteins with and without myristoylation. As reported (15), the intrinsic GTPase activity of the recombinant ARL1 (non-myristoylated) was higher than that of the ARFs (data not shown). Enhancement by ARF GAP was, however, easily demonstrated (Fig. 6). It seems likely that ARL activity, like that of ARF, is regulated by a GAP protein(s), whether or not by an ARL-specific GAP remains to be demonstrated. It is likewise unclear at present how many different ARF GAPs may exist. ARF GAP is alternatively spliced, and this processing may give rise to proteins with different substrate specificities and responses to phospholipids (25).

ARD1 was initially identified by cDNA cloning as a 64-kDa protein with an ~18-kDa ARF sequence at the COOH terminus (13). The NH\(_2\)-terminal part of ARD1 (p5) has been shown recently to function as a GAP for the COOH-terminal ARF domain (p3) (14). The ability of p5 to enhance the GTPase activity of ARL1 and several ARF proteins was, therefore, investigated (Table II). Although recombinant p5 markedly increased GTP hydrolysis by p3, as already reported (14), no GAP activity toward native or recombinant ARF proteins or ARL1 was detected. It appears that the NH\(_2\)-terminal portion of ARD1 serves specifically as a GAP for the ARF domain of that protein. This is in marked contrast to the apparently rather broad substrate specificity of the ARF GAP purified from spleen cytosol. Consistent with these data, amino acid sequences of the GAP domain of ARD1 (13) and of peptides from spleen ARF GAP2 were not notably similar, nor were those of the peptides and the deduced amino acid sequence of a rat liver ARF GAP cloned by Cukierman et al. (25). The meaning of this, at the moment, seemingly negative, information will remain unclear until we are able to demonstrate directly that the peptide sequence is part of a protein that exhibits GAP activity.

Acknowledgments—We thank Drs. Walter Patton, Jin-Xin Hong, and Guifang Zhang for providing recombinant ARF and ARL proteins and Carol Kosh for secretarial assistance.

REFERENCES

1. Kahn, R. A., and Gilman, A. G. (1984) J. Biol. Chem. 259, 6228–6234
2. Tsai, S.-C., Noda, M., Adamanik, R., Chang, P. P., Chen, H.-C., Moss, J., and Vaughan, M. (1988) J. Biol. Chem. 263, 1768–1772
3. Moss, J., and Vaughan, M. (1995) J. Biol. Chem. 270, 12327–12330
4. Kahn, R. A., Yuetel, J. K., and Malhotra, V. (1993) Cell 75, 1045–1048
5. Kende, G. A., Gutowski, S., Moenaw, C., Slaughter, C., and Sterneck, P. C. (1993) Cell 75, 1137–1144
6. Cockcroft, S., Thomas, G. M. H., Geny, B., Cunningham, E., Geut, J., Hiles, J., Toty, N. F., Truong, O., and Hsuan, J. J. (1994) Science 263, 525–526
7. Sewell, J. L., and Kahn, R. A. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4620–4624
8. Price, S. R., Nightingale, M. S., Tsai, S.-C., Williamson, K. C., Adamanik, R., Chen, H.-C., Moss, J., and Vaughan, M. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5488–5491
9. Babak, D. A., Nightingale, M. S., Murtagh, J. J., Price, S. R., Moss, J., and Vaughan, M. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 6101–6105
10. Monaco, L., Murtagh, J. J., Newman, K. B., Tsai, S.-C., Moss, J., and Vaughan, M. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2206–2210

* M. Ding, N. Vitale, S.-C. Tsai, R. Adamanik, J. Moss, and M. Vaughan, unpublished observations.
Characterization of a GTPase-activating Protein That Stimulates GTP Hydrolysis by Both ADP-ribosylation Factor (ARF) and ARF-like Proteins: COMPARISON TO THE ARD1 GAP DOMAIN
Min Ding, Nicolas Vitale, Su-Chen Tsai, Ronald Adamik, Joel Moss and Martha Vaughan

J. Biol. Chem. 1996, 271:24005-24009.
doi: 10.1074/jbc.271.39.24005

Access the most updated version of this article at http://www.jbc.org/content/271/39/24005

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 28 of which can be accessed free at http://www.jbc.org/content/271/39/24005.full.html#ref-list-1