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Differential interaction of ADP-ribosylation factors 1, 3, and 5 with rat brain Golgi membranes

(cholera toxin/protein trafficking/endoplasmic reticulum/vesicular transport)

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ABSTRACT Six mammalian ADP-ribosylation factors (ARFs) identified by cDNA cloning were expressed as recombinant proteins (rARFs) that stimulated cholera toxin ADP-ribosyltransferase activity. Microsequencing of soluble ARFs I and II (sARFs I and II), purified from bovine brain, established that they are ARFs 1 and 3, respectively. Rabbit antibodies (IgG) against sARF II reacted similarly with ARFs 1, 2, and 3 (class I) on Western blots. ARFs 1 and 3 were distinguished by their electrophoretic mobilities. Antiserum against rARF 5 cross-reacted partially with rARF 4 but not detectably with rARF 6 and minimally with class I ARFs. Guanosine 5′-*O*-(3-thiotriphosphate) (GTP[γS]) increased recovery of ARF activity and immunoreactivity in organelle fractions separated by density gradient centrifugation, after incubation of rat brain homogenate with ATP and a regenerating system. ARF 1 accumulated in microsomes plus Golgi and Golgi fractions, whereas ARF 5 seemed to localize more specifically in Golgi; the smaller increment in ARF 3 was distributed more evenly among fractions. On incubation of Golgi with a crude ARF fraction, GTP[γS], and an ATP-regenerating system, association of ARF activity with Golgi increased with increasing ATP concentration paralleled by increases in immunoreactive ARFs 1 and 5 and, to a lesser degree, ARF 3. Golgi incubated with GTP[γS] and purified ARF 1 or 3 bound more ARF 1 than ARF 3. Based on immunoreactivity and assay of ARF activity, individual ARFs 1, 3, and 5 appeared to behave independently and selectively in their GTP-dependent association with Golgi *in vitro*.

Mammalian ADP-ribosylation factors (ARFs), 20-kDa GTP-binding proteins, are products of at least six ARF genes (1–6). The recombinant proteins expressed in *Escherichia coli* exhibit the characteristic GTP-dependent stimulation of cholera toxin A subunit (CTA)-catalyzed ADP-ribosylation (7). Their deduced amino acid sequences contain consensus sequences that are believed to be involved in guanine nucleotide binding and GTP hydrolysis (8). Based on size, deduced amino acid sequence, and gene structure, they have been divided into three classes: class I, ARFs 1, 2, and 3; class II, ARFs 4 and 5; class III, ARF 6 (2, 3, 6, 9). They are ubiquitous, highly conserved proteins distributed widely in eukaryotes from *Giardia* to mammals (10–12).

ARF was originally identified as a factor required for the GTP-dependent cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ (stimulatory regulatory protein of adenylyl cyclase) (1, 13–17). Recently, ARFs have been implicated in protein transport in the Golgi (18–20). In normal rat kidney cells, during translocation of non-clathrin-coated vesicles in the presence of the nonhydrolyzable GTP analogue guanosine 5′-*O*-(3-thiotriphosphate) (GTP[γS]) (20), ARF accumulated in the Golgi, as shown by immunofluorescence (20). Immunofluorescence also demonstrated accumulation of β-COP, one of the subunits of a non-clathrin complex termed coatomer (20, 21). After *in vitro* incubation of a Golgi fraction with cytosol and GTP[γS], ARF and β-COP were recovered with the Golgi (20). It was shown also that brefeldin A, a fungal product, which causes dispersion of the Golgi into tubular structures and dissociation of β-COP, prevented association of β-COP and ARF with Golgi membranes (20).

In the studies reported here, ARFs were identified by their activity in accelerating cholera toxin-catalyzed ADP-ribosylation of agmatine (16, 17), by immunoreactivity with specific antibodies, and by electrophoretic mobility. Three of the family of six mammalian ARFs thus far known are shown directly to interact with rat brain Golgi and perhaps other membranes *in vitro*.

MATERIALS AND METHODS

Preparation of 850 × *g* Supernatant and Golgi Fraction from Rat Brain Homogenate. Fresh rat brain (44 g) was minced and homogenized in a Teflon Dounce homogenizer (six strokes) in 88 ml of buffer A [0.25 M sucrose/1 mM $MgCl_2$/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride (PMSF)/soybean and lima bean trypsin inhibitors, leupeptin, and aprotinin, each 1 μg/ml/10 mM Hepes buffer, pH 7.5] (22). The homogenate was diluted with 88 ml of buffer A and centrifuged (850 × *g*, 10 min, Sorval SS 34, 2760 rpm). The supernatant either was used directly (Table 2) or was centrifuged for 35 min at 175,000 × *g* (SW 41, 37,000 rpm) to prepare Golgi. The pellet was gently dispersed in buffer A containing 1.4 M sucrose (40 ml). A sample (4 ml) was transferred to the bottom of a centrifuge tube that contained layers (each 2 ml) of 0.25, 0.6, 0.85, and 1.15 M sucrose in buffer A without sucrose and protease inhibitors. After centrifugation (175,000 × *g*, 100 min, SW 41, 37,000 rpm), the cellular organelles concentrated at the interfaces of the sucrose layers were collected.

Fractions were assayed for activity of galactosyltransferase (23), a trans Golgi enzyme. Fraction 5 (0.85/1.15 M sucrose, designated Golgi) exhibited the highest specific activity. Fractions 4 and 6 (0.6/0.85 and 1.15/1.4 M sucrose, designated as mixtures of Golgi and microsomes and Golgi, mitochondria, and plasma membranes, respectively) also contained significant amounts of activity. Fraction 5 was stored in small portions at −70°C until used for Golgi experiments.

Interaction of ARF with Golgi. Samples of Golgi (50 μg of protein) were incubated at 37°C with additions as indicated in buffer B (90 mM NaCl/4.4 mM $MgCl_2$/0.8 mM EDTA/10 mM DTT/0.25 M sucrose/18 mM Tris, pH 8.0) (total volume,

Abbreviations: ARF, ADP-ribosylation factor; CTA, A subunit of cholera toxin; PMSF, phenylmethylsulfonyl fluoride; rARF, recombinant ARF; sARF I and sARF II, two soluble ARFs from bovine brain; GTP[γS], guanosine 5′-*O*-(3-thiotriphosphate); GDP[βS], guanosine 5′-*O*-(2-thiodiphosphate).

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450 μl) and then centrifuged (16,000 × g, 30 min) at 4°C. Supernatant was removed, the inside of the tube was blotted, and 200 μl of buffer A was added gently to rinse the pellet. Finally, the pellet was dispersed in buffer A (200 μl) by pipetting. A sample (20 μl, 5 μg of protein) was used for determination of protein, 20- and 40-μl samples were assayed for ARF activity—i.e., activation of CTA ADP-ribosyltransferase activity—and a 100-μl sample (≈25 μg of protein) was used for immunodetection of ARFs 1, 3, and 5.

Preparation of Brain ARF Fraction. Rat brain supernatant (175,000 × g, 35 min) was concentrated to half its volume (≈15 mg of protein per ml), and an 18-ml sample was applied to a column (2 × 89 cm, 280 ml) of Ultrogel AcA 54 equilibrated and eluted with buffer C (0.25 M sucrose/100 mM NaCl/1 mM NaN_3/5 mM $MgCl_2$/1 mM EDTA/2 mM DTT/20 mM Tris, pH 8.0). Fractions (3 ml) were collected and assayed for stimulation of CTA NAD:agmatine ADP-ribosyltransferase activity. Fractions that increased CTA activity were pooled, concentrated to ≈2 mg of protein/ml, and used as brain ARFs.

Preparation of Recombinant ARF (rARF) Proteins. rARF 2 and rARF 6 were prepared as described (7). Sequences encoding human ARFs 3, 4, and 5 were cloned into the expression vector pGEX-5G/LIC by a ligation-independent cloning procedure (24). ARF 4 sequences were amplified using the primers 5′-GGCCTGGTTCCGCGGGCCTCACTATC-3′ and 5′-CTGCGCCTCGCTCCAATTTCATTTAA-3′ (underlined nucleotides correspond to ARF 4 cDNA sequences) and an ARF 4 cDNA clone as template. ARF 3 and 5 expression vectors were prepared and fusion proteins were purified and cleaved with thrombin as described (7).

Immunodetection of ARFs 1, 3, and 5. Preparation of rabbit anti-sARF II IgG and procedures for development of immunoblots have been described (sARF I and sARF II are soluble ARFs from bovine brain; ref. 11). rARF 5 antiserum was obtained from a rabbit immunized with rARF 5 synthesized in *E. coli* from human ARF 5 cDNA (7, 24). Samples of proteins were subjected to SDS/polyacrylamide gel electrophoresis in 14% gels (25, 26) and transferred to nitrocellulose paper (27). Some blots were incubated with rARF 5 antiserum (diluted 1:1000), then washed, and incubated with anti-sARF II IgG, 0.5 μg/ml. Depending on the intensity of reaction, the blots were allowed to react a second time with rARF 5 antibodies.

Other Assays. Measurement of ADP-ribosylagmatine formation was carried out as described (16, 17) with the addition of cardiolipin (1 mg/ml), 500 μM ATP, and 60 μM Cibachrome blue F3G-A (Fluka). Cibachrome blue, which inhibited >85% of NADase activity in Golgi, was required to achieve a linear relationship between the amount of ARF and the increment in toxin-catalyzed ADP-ribosylagmatine synthesis. Specific activity of NAD was 3200–3400 cpm/nmol. Total radioactivity of samples without CTA was 300–400 cpm and of samples with 1 μg of CTA, 1100–1400 cpm. Activity with CTA alone was subtracted for calculation of ARF activity.

Protein was measured by Bio-Rad assay with bovine serum albumin as standard. Membranes were incubated in 0.1 M NaOH at ≈65°C for 10 min, before samples were taken for protein assay.

Sources of materials not indicated here are noted in earlier publications from this laboratory.

Table 1. Comparison of peptide and deduced amino acid sequences (169–178) of class I ARFs

Protein	Amino acids 169–178	Source of sequence
ARF 1	GLDWLSNQLR	Deduced (ref. 2)
	GLDW-S-QL-	sARF I peptide
ARF 2	GLDWLSNQLK	Deduced (ref. 4)
ARF 3	GLDWLANQLK	Deduced (ref. 2)
	GLDW-ANQ-K	sARF II peptide

Amino acid sequences of peptides resulting from trypsin and CNBr hydrolysis, respectively, of sARF I and sARF II purified from bovine brain cytosol (17) were determined by the Harvard Microchem Laboratory (Cambridge, MA) and H.-C. Chen (National Institute of Child Health and Human Development, National Institutes of Health) (4).

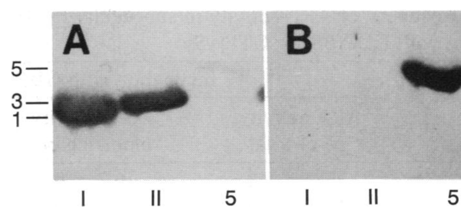

FIG. 1. Reaction of sARF I, sARF II, and rARF 5 with antibodies against sARF II and rARF 5. Samples of sARF I (I) and sARF II (II), each 1 μg, and of rARF 5 (0.25 μg) were subjected to electrophoresis in 14% gels and transferred to nitrocellulose in duplicate. (*A*) Blot incubated with anti-sARF II IgG, 1 μg/ml. (*B*) Blot incubated with rARF 5 antibodies (whole serum diluted 1:1000).

RESULTS AND DISCUSSION

We earlier purified two soluble ARFs, sARF I and sARF II, from bovine brain (17). Based on amino acid sequences of peptides from these proteins, sARF I has now been identified as a product of the ARF 1 gene and sARF II as that of the ARF 3 gene (Table 1). The latter had been suspected when it was found that sARF II increased in rat brain during postnatal development, whereas, among mRNAs for the six known ARFs, only that for ARF 3 increased in parallel (11). It may be noted that the sequences of the peptides in Table 1 should permit distinction of ARF 2 from ARFs 1 and 3. These identifications are based on the assumption that mammalian ARFs with identical sequences in this region but differences elsewhere do not exist.

Anti-sARF II IgG reacted apparently equally well with ARF 1 (sARF I) and ARF 3 (sARF II), which differed in electrophoretic mobility (Fig. 1). rARF 2 reacted similarly but we have not identified ARF 2 in adult rat brain. The IgG cross-reacted relatively slightly with rARF 4 (Fig. 2) and rARF 5 (Fig. 1). No ARF 4 was detected in rat brain in these experiments. Anti-rARF 5 antibodies reacted with rARF 5 and, to a lesser extent, with rARF 4, which, however, could be separated electrophoretically from ARF 5 (data not shown). Rat brain ARF 5 separated from other ARFs by

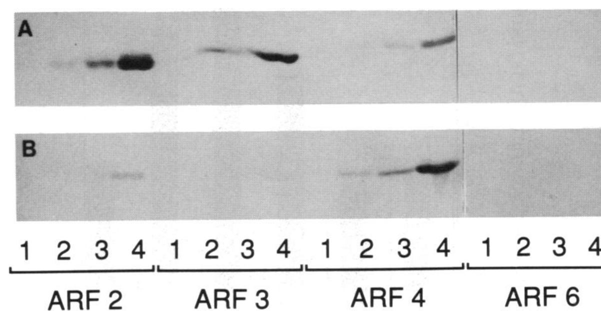

FIG. 2. Reaction of rARFs 2, 3, 4, and 6 with antibodies against sARF II and rARF 5. Samples of rARFs were subjected to electrophoresis in 15% gels and transferred to nitrocellulose blots in duplicate. (*A*) Blot incubated with anti-sARF II IgG, 1 μg/ml. (*B*) Blot incubated with rARF 5 antibodies (whole serum diluted 1:1000). Lanes 1, 2, 3, and 4 contained 0.094, 0.19, 0.38, and 0.94 μg of protein, respectively.

Table 2. Recovery of ARF activity in subcellular fractions separated by sucrose density centrifugation after incubation of 850 × *g* supernatant with GDP[βS] or GTP[γS]

	Incubation with GDP[βS]			Incubation with GTP[γS]		
Fraction	ARF activity, % of total	GT activity, nmol/mg per h	Protein, % of total	ARF activity, % of total	GT activity, nmol/mg per h	Protein, % of total
1 S1	73.2 ± 1.2	0.3 ± 0.2	52.7 ± 1.54	42.9 ± 1.4	1.1 ± 0.7	51.1 ± 1.5
2 S2	16.1 ± 1.0	1 ± 0.3	13.6 ± 0.0	12.0 ± 0.9	0.8 ± 0.4	14.0 ± 0.4
3 LMc	2.6 ± 1.3	0.4 ± 0.03	5.0 ± 0.4	9.3 ± 0.6	0.4 ± 0.3	5.7 ± 0.3
4 Mc/G	1.5 ± 0.3	4.8 ± 1.4	2.9 ± 0.4	7.2 ± 0.1	2.8 ± 0.3	3.4 ± 0.1
5 G	2.1 ± 0.1	11.1 ± 2.5	4.6 ± 0.1	10.6 ± 0.5	10.4 ± 1.0	5.6 ± 0.2
6 G/Mt/PM	4.2 ± 0.5	3.3 ± 0.6	20.5 ± 0.2	17.7 ± 1.1	4.7 ± 0.5	19.9 ± 2.0
Total mg			49.7 ± 1.9			49.2 ± 2.7
Total units	3452 ± 264			4177 ± 135		

Triplicate 3.8-ml samples of the 850 × *g* supernatant were incubated at 37°C for 40 min with an ATP-regenerating system (5 mM creatine phosphate/creatine phosphokinase, 10 units/ml), 1 mM ATP, and 200 μM GDP[βS] or GTP[γS]. After incubation, each sample was transferred to the top of a tube containing 1.5-ml layers of 0.4, 0.6, 0.85, and 1.15 M sucrose in buffer A and 2 ml of 1.4 M sucrose in buffer A, which was centrifuged for 105 min (175,000 × *g*, SW 41, 37,000 rpm). Six fractions were collected from each tube (from the top): 1, 3.3 ml; 2, 1.3 ml; and 3–6, 1.5 ml. Samples of fractions were assayed for ARF activity, galactosyltransferase, and protein. One unit of ARF activity equals 1 nmol/h. Data are means ± SEM of values from three samples. This experiment was replicated four times. GT, galactosyltransferase; S1 and S2, supernatants 1 and 2; LMc, light microsomes; Mc/G, microsomes/Golgi; G, Golgi; G/Mt/PM, Golgi, mitochondria, and plasma membrane.

DEAE-Sephacryl chromatography exhibited characteristic activation of CTA-catalyzed ADP-ribosylation (data not shown).

As class I (ARFs 1, 2, and 3) and class II (ARFs 4 and 5) ARFs differ about 20% in overall sequence, with greater degrees of difference in the C-terminal one-half and the N-terminal 17 amino acids, it is perhaps not surprising that the antibodies prepared against ARF 3 (sARF II) and rARF 5 are relatively specific for class I and class II ARFs, respectively. ARFs 1, 2, and 3 (>95% identical) reacted similarly with anti-sARF II IgG. ARF 4, which differs by 10% from ARF 5, was somewhat less reactive with rARF 5 antibodies. ARF 6 protein, which did not react with either set of antibodies (Fig. 2), has not been identified in any tissue, although mRNA has been detected (6, 11).

After incubation of the 850 × *g* supernatant with ATP, an ATP-regenerating system, and GTP[γS] or guanosine 5′-*O*-(2-thiodiphosphate) (GDP[βS]) (20) at 37°C for 40 min, ARF activity in sucrose density gradient fractions 3–6 in the GTP[γS] samples (relative to the GDP[βS] samples) was increased, roughly corresponding to the decrement in activity in fractions 1 and 2, although total recovery of ARF activity was somewhat higher in samples incubated with GTP[γS], as was total galactosyltransferase activity (Table 2). A similar effect of GTP[γS] on immunoreactive ARF in PC-12 cell homogenates was attributed to stabilization (25), but effects of membrane phospholipids on toxin ADP-ribosyltransferase activity in different fractions could also contribute in this experiment. Differences in stability of individual ARFs may also be a factor, as only class I ARFs were detected in the PC-12 cell studies, whereas all ARFs are detected in the activity assays. Total recoveries of protein in GDP[βS]- and GTP[γS]-treated samples were identical. After incubation with GTP[γS], ARF activity in fractions 3–6 was three to five times that after incubation with GDP[βS] (Fig. 3). It will be noted (Table 2) that the ratio of ARF activity to galactosyltransferase activity differs greatly in different fractions, perhaps because specific ARFs associate with membranes (Golgi or other) different from those that contain galactosyltransferase.

During incubation of rat brain homogenate with GTP[γS], but not GDP[βS], immunoreactive ARFs 1 and 5 were accumulated in fractions 4 (microsomes plus Golgi) and 5 (Golgi) more than in fractions 3 (light microsomes) and 6 (Golgi, mitochondria, and plasma membrane) (Fig. 4). ARF 5 concentrated in fraction 5 more than in fraction 4, whereas ARF 1 appeared to associate equally with fractions 4 and 5. Although ARF 3 (sARF II) was much more abundant than ARF 1 in rat brain (11), after incubation with GTP[γS] the intensity of the ARF 3 band was increased only slightly in fractions 3–6, clearly not as much as ARF 1 (or ARF 5). Little or no ARF 1, 3, or 5 was detectable in membrane fractions from GDP[βS]-treated samples.

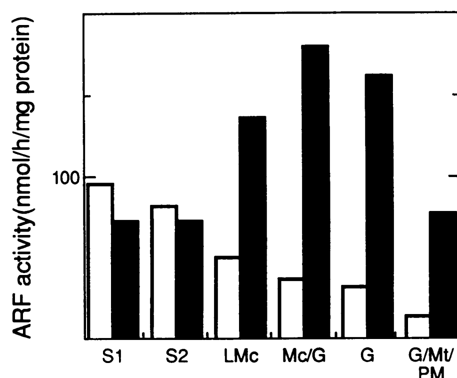

FIG. 3. Effect of GTP[γS] on recovery of ARF activity in subcellular fractions. Data are from the experiment shown in Table 2. Specific activity is expressed in nmol of ADP-ribosylagmatine formed per h per mg of protein in each fraction. Fractions 1–6 are identified as in Table 2. □, Incubated with GDP[βS]; ■, incubated with GTP[γS]. The experiment was replicated four times.

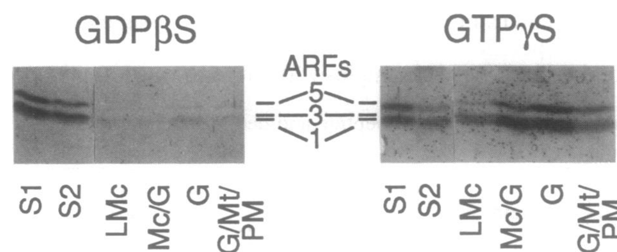

FIG. 4. Reaction of anti-sARF IgG and rARF 5 antiserum with ARFs 1, 3, and 5 in subcellular fractions. Proteins (50 μg) from each sucrose density gradient fraction were precipitated with 7.5% trichloroacetic acid, dissolved in SDS/mercaptoethanol, separated by electrophoresis in 14% gels (26), and transferred to nitrocellulose. Blots were incubated with anti-sARF II IgG (1 μg/ml) then incubated twice with anti-rARF 5 antiserum (diluted 1:1000), and developed. (*Left*) Samples from incubation with 200 μM GDP[βS]. (*Right*) Samples from incubation with 200 μM GTP[γS]. Lanes are identified as in Table 2.

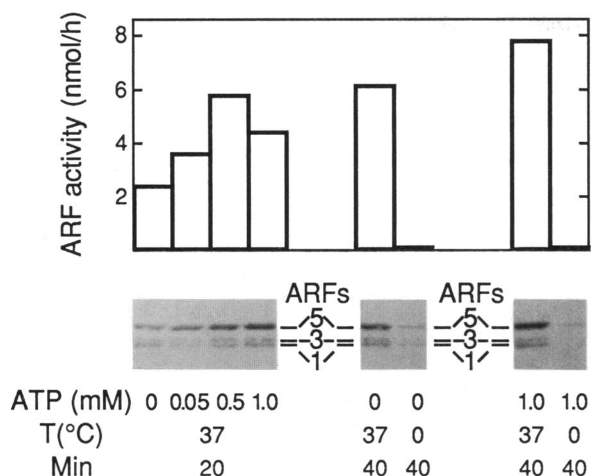

FIG. 5. Effect of ATP on ARF activity and immunoreactive ARFs 1, 3, and 5 in Golgi. Samples of Golgi proteins (50 μg) and rat brain ARFs (64 μg) pooled from Ultrogel AcA 54 column chromatography were incubated at 0°C or 37°C for 20 or 40 min with 50 μM GTP[γS]/5 mM creatine phosphate/5 units of creatine phosphokinase/90 mM NaCl/4.4 mM $MgCl_2$/0.8 mM EDTA/10 mM dithiothreitol/0.25 M sucrose/18 mM Tris buffer, pH 8/ATP, as indicated. (*Upper*) ARF activity in Golgi (nmol/h). (*Lower*) Reaction of ARFs with anti-sARF II IgG and anti-rARF 5 antiserum on Western blots.

On incubation of Golgi with a crude ARF fraction from rat brain, GTP[γS], and an ATP-regenerating system, association of ARFs with Golgi increased with increasing ATP concentration (Fig. 5). There was an increment in ARF activity and immunoreactive ARF in Golgi between 20 and 40 min in the presence of 1 mM ATP and no detectable ARF–Golgi interaction at 0°C (Fig. 5). Data do not permit distinction between an effect of ATP on rate or extent of ARF interaction with Golgi. As observed after incubation of homogenate with GTP[γS] (Fig. 4), in this somewhat more simplified system, accumulation in the Golgi fraction of ARF 5 and, to a lesser extent, of ARF 1 was more prominent than that of ARF 3 (Fig. 5).

Similarly, Golgi incubated with GTP[γS], ATP with regenerating system, and 10 μg of purified ARF 1 or ARF 3, bound more ARF 1 than ARF 3 based on ARF activity associated with Golgi (19.4 vs. 10.4 nmol/h). Doubling the amount of added ARF increased the amount of ARF 1 bound (19.4 to 24.4 nmol/h) but not that of ARF 3 (10.4 vs. 10.6 nmol/h). It seems rather impressive that ARF 1 and ARF 3, which differ in only 7 of 181 amino acids, are apparently so selectively bound. Three of the differences are, however, among the 8 C-terminal amino acids, which perhaps makes those regions sufficiently distinctive to assure specificity of recognition. It was demonstrated by expression of *rab* 2, *rab* 5, and *rab* 7 hybrid proteins in baby hamster kidney cells that the C-terminal 34 amino acids of these proteins (which overall differ more than do the ARFs) contain the information necessary for correct targeting (28). The extreme C-terminal cysteine sequence (which differs among the *rabs*) is required for membrane association, presumably because it is isoprenylated (29), but does not determine specificity. The ARF proteins lack similar C-terminal cysteines (or upstream cysteines that are palmitoylated, as in the *ras* proteins) (8). Thus, the role of the ARF C terminus in membrane association remains to be defined.

Recently published evidence (30) clearly establishes the importance of the N terminus (some or all of N-terminal 17 amino acids) in the activity of ARF as an activator of cholera toxin or in the formation of Golgi-derived vesicles but does not address the question of specificity. Myristoylation of the N-terminal glycine (10), which may promote assumption of an α-helical conformation by the N terminus of ARF (30), surely contributes to membrane interaction, if not to specificity. The deduced sequences of all ARF proteins contain a glycine adjacent to the initiating methionine, and the ARFs are, therefore, potential substrates for *N*-myristoyltransferase, although differences in adjacent sequence may influence their suitability (31). The change in protein conformation that results from replacement of bound GDP with GTP increases ARF binding to phospholipid (25, 32), again presumably without contributing to specificity of membrane interaction. On the other hand, ARFs do interact preferentially with certain lipids (25), and differences in membrane lipid composition—e.g., ceramide concentrated in trans Golgi in some cells (33)—could contribute to specificity of interactions. Nevertheless, an ARF "target" seems generally to be viewed as a protein, perhaps a specific GTPase-activating protein that accelerates ARF GTP hydrolysis, thereby initiating the next step in the sequence (19).

Because the class I ARFs are so similar to each other (95–96% amino acid identity) it seems likely that functional specificity is achieved, at least in part, by regulated expression of these proteins in different cells or at different times in cellular development. Interpretation of the observations reported here is thus necessarily limited because of the use of a heterogeneous population of cells as a source of membranes and, in some experiments, of ARFs also. To define precisely the functions of the individual ARFs it will be necessary to use homogenous populations of cells, more rigorous separation and characterization of cellular organelles (membranes), and new antibodies that will permit identification and quantification of the individual ARF proteins. It should then be possible to define better the specific roles of the six known mammalian ARFs, remembering that, like the *rab* proteins, some might function in an endocytic rather than an exocytic pathway.

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