ADP-ribosylation factors (ARFs) are 20-kDa guanine nucleotide-binding proteins, members of the Ras GTPase superfamily that were initially recognized and purified because of their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A subunit (CTA). We now know that they are critical components of several different vesicular trafficking pathways in all eukaryotic cells and activators of specific phospholipidase Ds (PLDs) (reviewed in Refs. 1–3). ARF interacts with many proteins and other molecules that regulate its state of activation or are involved in its intracellular function (Fig. 1).

As with other members of the Ras superfamily, ARF with GDP bound is inactive. Replacement of bound GDP with GTP produces active ARF-GTP, which can associate with membranes. ARF-GTP is the form that activates CTA and PLD. Both forms are important in vesicular transport, which requires that the ARF molecule cycle between active and inactive states. Like the many other GTP-binding proteins or GTPases that are molecular switches for the selection, amplification, timing, and delivery of signals from diverse sources, ARF functions via differences in conformation that depend on whether GTP or GDP is bound. Vectorial signaling results from the necessary sequence of GTP binding, hydrolysis of bound GTP, and release of the GDP product (Fig. 2).

Under physiological conditions, release of GDP from ARF, the prerequisite for GTP binding, is very slow and is markedly accelerated by guanine nucleotide-exchange proteins or GEPs, several of which are now known. Hydrolysis of bound GTP to yield ARF-GDP, i.e. the inactivation or "turn-off" reaction, is similarly very slow (undetectable) in the absence of specific GTPase-activating proteins or GAPs. During the last 3 years, a large part of the new information related to ARF structure and function concerns these two major types of ARF regulatory proteins, which are the subjects of this review. In addition, the relatively small ~180-amino acid ARF proteins interact with numerous other molecules (not all simultaneously). These include CTA, PLD, and guanylyl nucleotide, of course, as well as PIP₂ (4), coatomers (5), arfaptin (6), G protein βγ subunits (7, 8), and Gα₉ (7), about which information is much more limited (Fig. 1). Self-association of ARF in functional dimers or tetramers has also been suggested (5), and the crystal structure of a dimer is published (9).

Criteria for designation as an ARF have been the ability to activate cholera toxin and to rescue mutant *Saccharomyces cerevisiae* bearing the lethal double deletion of ARF¹ and ARF² genes. ARF-like proteins (ARLs), which are structurally very similar to ARFs, were initially believed not to possess these ARF activities. It is now known, however, that under suitable assay conditions, at least some ARLs can exhibit ARF activity (10), and there is perhaps a continuum of ARF-ARL function. Neither of the two criteria may, in fact, reflect accurately the properties that are required for physiological ARF function in vesicular trafficking or PLD activation. The mutant yeast rescue assay seems ambiguous, because diverse ARFs from many species can be effective when overexpressed, although yeast ARF₃, which resembles most closely mammalian ARF₆, is ineffective (11). In the assay of CTA (or PLD or guanine nucleotide binding), dramatic effects of specific phospholipids and detergents as well as concentrations of Mg²⁺ and salt on the activities of individual ARFs are well known and make it difficult to draw valid inferences about the functional relevance of many in vitro observations.

Mammalian ARFs are divided into three classes based on size, amino acid sequence, gene structure, and phylogenetic analysis; ARF¹, ARF², and ARF³ are in class I, ARF₄ and ARF₅ are in class II, and ARF₆ is in class III (3). Non-mammalian class I, II, and III ARFs have also been found (3). A role for class I ARFs 1 and 3 in ER to Golgi and intra-Golgi transport is well established (1, 2). ARF₆ has been implicated in a pathway involving plasma membrane and a tubulovesicular compartment that is distinct from previously characterized endosomes (12, 13).

Vesicular transport has been extensively studied in the Golgi and ER to Golgi pathways (1, 2). The mechanisms, including the molecules and their functions, are likely very similar in other pathways. Formation of a transport vesicle begins when activated ARF with GTP bound associates with the cytoplasmic surface of a donor membrane. Just how the initiation site is identified remains unknown. Activated ARF interacts with a coat protein, one of seven in the coatamer complex. Recruitment of multiple ARF molecules followed by coatamer causes membrane deformation and budding. Bilayer fusion at the base of a bud induced by fatty acyl-CoA results in vesicle release. Roles for PLD in both vesicle formation (14) and fusion (15) have been suggested. Removal of the coat, which is necessary for vesicle fusion at the target membrane, requires inactivation of ARF by hydrolysis of bound GTP to GDP. This description is necessarily greatly oversimplified. There is no consideration of the many other molecules, protein and lipid, in each transport vesicle that have structural, metabolic, or signaling functions.

*Fig. 1. Molecules in the ARF orbit. ARF interacts with three general types of molecules: Regulatory proteins GAP and GEP (Fig. 2) are discussed in the text. Arfaptins (6) are ~44-kDa proteins identified in a yeast two-hybrid screen of a HL-60 cDNA library using dominant active ARF³ (Q71L) as bait. The recombinant GST-arfaptin fusion protein bound activated recombinant human ARFs 1, 3, 5, and 6 to an extent decreasing in that order. The possibility that the complex of activated ARF and arfaptin is a functional entity remains to be explored. Small non-protein molecules like guanyl nucleotides and PIP₂, with specific binding sites have major effects on ARF conformation and, therefore, activity. PLD and coatomer are two major effectors with which ARF interacts. The functional significance of its demonstrated interaction with G protein βγ subunits (7, 8) is unknown. This minireview will be reprinted in the 1998 Minireview Compendium, which will be available in December, 1998. This is the third article of five in the "Small GTPases Minireview Series." To whom correspondence should be addressed: Rm. 5N-307, Bldg. 10, 10 Center Dr. MSC 1434, National Institutes of Health, Bethesda, MD 20892-1434. Tel.: 301-496-4554; Fax: 301-402-1610; E-mail: vaughanm@gwgate.nih.gov.

*This minireview will be reprinted in the 1998 Minireview Compendium, which will be available in December, 1998. This is the third article of five in the "Small GTPases Minireview Series." To whom correspondence should be addressed: Rm. 5N-307, Bldg. 10, 10 Center Dr. MSC 1434, National Institutes of Health, Bethesda, MD 20892-1434. Tel.: 301-496-4554; Fax: 301-402-1610; E-mail: vaughanm@gwgate.nih.gov.

The abbreviations used are: ARF, ADP-ribosylation factor; ARL, ARF-like protein; CTA, cholera toxin A subunit; PLD, phospholipase D; ER, endoplasmic reticulum; PIP₂, phosphatidylinositol 4,5-bisphosphate; GEP, guanine nucleotide-exchange protein; GAP, GTPase-activating protein; PH, pleckstrin homology; ARNO, ARF nucleotide-binding site opener; BFA, brefeldin A; PC, phosphatidylcholine; GST, glutathione S-transferase; GTPγS, guanosine 5′-O-(3-thiotriphosphate); GDPβS, guanosine 5′-O(2-thiodiphosphate); GRP1, general receptor for phosphoinositides.
Guanine Nucleotide-exchange Proteins

ARF GEPs exist in soluble and particulate forms, some of which can be inhibited by brefeldin A (BFA). BFA is a fungal fatty acid metabolite that reversibly blocks protein secretion and causes disintegration of Golgi structure in many cells by blocking GEP-catalyzed ARF activation (16, 17). Both BFA-sensitive and -insensitive GEPs have been purified and cloned. The deduced amino acid sequence of a soluble ~200-kDa BFA-sensitive GEP that had been purified from brain contains a so-called Sec7 domain (18). Assays of a series of deletion mutants established that the Sec7 domain itself has BFA-sensitive ARF GEP activity. This is modulated, however, by elements nearer the N terminus that increase catalytic activity ~100-fold and also increase BFA sensitivity. Several smaller (~50 kDa) GEPs also possess Sec7 domains (19) but are not inhibited by BFA. These include two proteins purified from bovine brain and spleen cytosol (20, 21) and recombinant ARNO (22, 23), cytohesin-1 (24), and GRP1 (25).

Sec7 is an essential yeast gene involved in protein secretion. The gene product is a ~230-kDa phosphoprotein that moves between membrane and cytosolic fractions (26). Two different Sec7 mutants were rescued in an allele-specific fashion by overexpression of human ARF4 and yeast ARF1 or yeast ARF2 (27). BFA inhibition of GEP activity of the Sec7 domain has recently been demonstrated (28). Two other ~160-kDa yeast proteins with Sec7 domains, Gea1 and Gea2, which are ~50% identical in deduced amino acid sequence, also display ARF GEP activity that is inhibited by BFA (29).

The ~50-kDa BFA-insensitive GEPs contain, in addition to the central Sec7 domain, an N-terminal coiled-coil region and a C-terminal pleckstrin homology (PH) domain that binds negatively charged phospholipids and is responsible for their ability to enhance GEP activity (22, 23). The most extensively studied of these is the 47-kDa ARNO. The GEP activity of its Sec7 domain toward myristoylated ARF1 was equal to that of intact ARNO but was unaffected by PI-Pi, which enhanced activity ~20-fold when the PH domain was present (22). PH domains are critical regions in nucleotide-exchange proteins for several Ras family GTPases as well as other regulatory molecules (briefly reviewed in Ref. 30).

Further studies defined in detail protein-phospholipid interactions that are involved in ARNO activation of ARF1 (29). With a mutant ARF1 lacking the first 17 amino acids as well as an N-terminal myristate (Δ17)ARF1, ARNO induced nucleotide exchange and PI-Pi had no effect, consistent with the conclusion that the PI-Pi-PH domain interaction is not involved in catalytic activity but acts to promote membrane association and interaction with the ARF substrate. Activation of intact myristoylated ARF1, on the other hand, depended on phospholipids and was favored by low concentrations of MgCl2 and salt (23).

PI-Pi is specifically required for several ARF actions, but the nature of the interaction was described relatively recently (4). In the crystal structure of ARF1-GDP (9), the N- and C-terminal α-helices are positioned in parallel on the surface of the molecule.

They contain the sites of interaction with PI-Pi, (Lys-15, Lys-16, Lys-181, and Arg-178), which were identified by mutation of single residues (4). These electrostatic interactions, which should be stronger at low ionic strength, along with hydrophobic interaction of the N-terminal myristate and membrane lipids, can account for the membrane association that is necessary for ARNO activation of ARF1 (29). Although details of the molecular mechanisms, i.e. the conformational changes and the sequence in which they occur, remain to be defined, it seems clear that acceleration of GDP release from ARF by ARNO is optimal when both proteins are interacting with phospholipids and the ARF N terminus is membrane-associated (23).

In experiments designed to assess ARNO interaction with ARF1 in different states of activation, the association of nucleotide-free (Δ17)ARF1 with the ARNO Sec7 domain (both soluble proteins) was shown by gel filtration (23). Attempts to compare ARNO affinities for GDP- and GTP-ligated myristoylated ARF1, although complicated by their very different affinities for membranes, led to the conclusion that ARNO probably has a higher affinity for ARF1-GDP (23).

The crystal structure of the ARNO Sec7 domain (23 kDa) shows a protein of 10 α-helices, the first seven of which are arranged in a right-handed superhelix with the last three forming a cap at one end (31). On one surface, there is a deep groove formed by two sequences of amino acids that are conserved among at least six Sec7 domains. The first of these (the longest identical sequence) contains a glutamate, mutation of which in EMB-30 abolished activity (19). The second sequence contains a high percentage of hydrophobic residues that are unusual in their exposure to solvent and were considered a likely locus of ARF interaction (31). When modeling of hydrophobic residues on the surface of the ARNO Sec7 domain molecule identified one area of high hydrophobicity that coincided with the postulated active site, a series of mutants with single amino acid replacements was studied. The conclusion was that Gly-155, Glu-156, and Met-194 are at the center of the active site and probably interact directly with ARF (31).

Having noted the observations of Paris et al. (23) that the myristoylated ARF N terminus interferes with ARNO stimulation of nucleotide exchange and that induction of a conformational change by phospholipid binding is necessary to permit ARNO action, Mosev et al. (31) used the soluble (Δ17)ARF1 to localize by protein “footprinting” the ARF sites that interact with ARNO. Two ARF regions protected from hydroxyl-radical cleavage were identified as the switch 1 (residues 41–55) and switch 2 (70–80) sequences. These are analogous in structure and function to regions in other 20-kDa GTPases that have been implicated in their interactions with specific GEPs. Because the sequences of the interacting regions are identical in mammalian ARFs 1–5 and differ in three of 26 positions in ARF6 (3), it was suggested that determinants of specificity may exist in other parts of the Sec7 domain proteins, although ARF specificity of the ARNO Sec7 domain was not itself assessed. The same switch regions participate in conformational changes induced by GAP activation of GTP hydrolysis, and it was pointed out (31) that this may complicate interpretation of some of the mutation data. ARNO induced a significant increase in the susceptibility of ARF to cleavage in a sequence involved in nucleotide binding (31). This would be consistent with evidence that GEPs stabilize the nucleotide-free state of the GTPase, e.g. the isolation of a complex containing nucleotide-free (Δ17)ARF1 and the ARNO Sec7 domain, which was reduced in amount when GDP was present (23).

Although ARF1 was used for all of the structural experiments with ARNO (31), Frank et al. (32) recently published studies leading to the conclusion that the brefeldin A-insensitive ARNO is a GEP for ARF6. They demonstrated its GEP activity in vitro toward ARF1 and ARF6 (both recombinant myristoylated proteins). When expressed in cultured cells, however, ARNO was localized by immunofluorescence microscopy and subcellular fractionation to the plasma membrane (32). This resembles the distribution of ARF6, which is associated with plasma membrane and tubulovesicular structures, is unaffected by BFA, and has been implicated in endocytosis and regulation of the actin cytoskeleton (12, 13). ARF1, on the other hand, functions in BFA-inhibited vesicular transport in the ER and Golgi (1–3). Hence, it was postulated that ARNO is
a BFA-insensitive GEP for ARF6, and a similar function was suggested for the 67% identical cytohesin-1 (32), which is present in relatively few cells (33), whereas ARNO is ubiquitous.2 BFA-insensitive GEP activity of cytohesin-1 toward native ARF1 and ARF3, but not recombinant myristoylated ARF5, had been reported earlier (24).

GRP1 (cytohesin-3) cDNA was cloned from mouse brain and adipocyte expression libraries in a search for proteins that bind 3'-phosphoinositides (25). It encodes a protein with Sec7 and PH domains 93 and 94% identical, respectively, to those in cytohesin-1 (25). Specific binding of phosphatidylinositol 3,4,5-trisphosphate by GRP1 and PH domains of cytohesin-1 and GRP1, but not other PH domains, was demonstrated. It was suggested that these proteins could link signaling pathways involving activation of phosphatidylinositol 3-kinases with those involving ARF and membrane trafficking or cell adhesion, based on the reported functional interactions of cytohesin-1 with β₂-integrin (34) as well as ARF (22–24).

Although there is infinitely more information about ARF GEPs than there was 3 years ago, there are now many more questions to be answered. Some are rather general and may be relatively easier to answer, e.g. do all ARF GEPs contain Sec7 domains and do all Sec7 domains have GEP activity? Other problems, however, such as the correct definition of physiological specificity of individual ARF GEPs are likely to require considerable work. It seems to be agreed that myristoylated ARFs are better than non-myristoylated ARFs as GEP substrates, presumably because of the contribution of an N-terminal myristate to membrane association, which facilitates the GEP interaction. Individual ARFs are affected differently by specific phospholipids and detergents, in terms of both guanine nucleotide binding and interactions with other proteins. Because ARFs are small proteins with rather limited differences in amino acid sequence, elucidation of the structural determinants of these characteristics may not be too distant. Such data could be useful in modifying GEP assays to reflect more accurately the ARF specificity that obtains in vivo.

GTPase-activating Proteins

Because GTP binding and GTP hydrolysis are equally critical to ARF functions in vesicular transport (and presumably other roles), it is unclear why so many more ARF GEPs than ARF GAPs (35–41) have been recognized and characterized. It may be due, in some part, to the use of assay conditions that are less than optimal. In addition, there might be, in fact, limited diversity among ARF GAPs, which would make them very different as a group from the much more numerous and structurally diverse Rho GAPs (42). At least one ARF GAP has apparently a very broad specificity, which includes mammalian ARFs 1, 3, 5, and 6 (independent of myristoylation) as well as ARL1 and ARF1 that lacks 13 amino acids at the N terminus (36). Perhaps that means that it can function at many sites in the cell, with the ARF substrate determined by GAP localization achieved by its interaction with specific intermediary proteins and/or phospholipids.

An apparently different ARF1 GAP purified and cloned from liver (37, 38) was recruited to membranes by overexpression of ERD2, a membrane receptor that recognizes the C-terminal sequence (Lys-Asp-Glu-Leu) found on certain soluble proteins (KDEL proteins) of the endoplasmic reticulum and serves to retrieve them if they are transported to the Golgi (39). Oligomerized ERD2 associated with the GAP, which then inactivated membrane-bound ARF and produced in the transfected cells a phenotype like that resulting from inhibition of ARF GEP (39). It was later shown that overexpression of lysozyme with a KDEL terminus, which was intended to increase engagement of the KDEL receptor in retrograde retrieval transport, increased its interaction with ARF GAP and ARF inactivation (43). This system nicely demonstrated a way in which vesicle content/cargo can influence a transport pathway (2).

In other experiments (40), diacylglycerol dramatically increased the activity of the recombinant GAP (amino acids 1–257). Because monounsaturated diacylglycerols are produced chiefly from PC via the sequential action of PLD and phosphatidate phosphohydrolase (whereas polyunsaturated diacylglycerols are derived from PIP₂ via phosphatidylinositol phospholipase C action), it was suggested that PLD activity could be a major regulator of ARF GAP (40). GAP activity was varied 100-fold by altering relative amounts of PC and diacylglycerol (40). Similar effects were observed on the activity of and lipid binding by Gcs1, an analogous ARF GAP from yeast (41). By comparing systematically the effects of phospholipid polar head groups and hydrocarbon chains on binding to the two GAPs, it was concluded that membrane association depended chiefly on hydrophobic interaction of the protein with hydrocarbon moieties of the lipid, which is favored by small head groups, and the conformation of monounsaturated acyl chains (40). In this view, the activation of ARF GAP results from increasing its concentration at the membrane where ARF-GTP resides. ARF activation of PLD leading to decreased PC and increased diacylglycerol levels would promote translocation of ARF GAP to a vesicle membrane where it could inactivate ARF-GTP and thereby terminate PLD action (40). The ARF GAPs that are activated by PIP₂ or other phosphoinositides (35, 38) are presumably subject to different kinds of regulation.

Lessons from ARD1

Studies with ARD1 (44), a 64-kDa protein that contains a C-terminal ARF sequence of ~18 kDa, and a region between amino acids 101–200 that acts as a GAP have also provided information about the structural requirements for this interaction (45–48). The ARF sequence referred to as p3, the remainder of the molecule (~46 kDa, p5), and the entire molecule (p8) were synthesized as GST fusion proteins and used for most studies after removal of the GST moiety. Both p8 and p3 activated CTA, and both bound GTP, but only p8 hydrolyzed it (45). Addition of p5 to p3 caused hydrolysis of GTP bound to p3. Separation of the GTP binding and GAP functions in the ARD1 molecule is reminiscent of a similar situation in α subunits of heterotrimeric G proteins (49).

The GAP activity of p5 was specific for p3 (36). Comparison of the amino acid sequences of p3 with those of ARFs that were not substrates revealed a striking difference in six of seven residues that correspond to the effector domain of Ras family proteins (ARF residues 39–45). Replacement of the seven amino acids in p3 (QDEFMQP) by the sequence from ARF1 (LGEIVTT), which is identical in all class I and class II ARFs, rendered it completely refractory to the effects of p5 (46). Conversely, ARF1 in which the LEIVTT had been replaced by the p3 sequence to produce ARF1 (39–45p3) was a p5 substrate. Single or double amino acid replacements demonstrated that aspartate and proline in the p3 sequence contributed to but did not completely for its role in the functional interaction with p5 (46).

Replacement of Cys109, Cys142, Cys147, and Cys152, anticipated to form a zinc finger structure in p5, showed that all were necessary for GAP activity but not for interaction with p3 (48). Similarly, in the cloned ARF1 GAP, replacement of either of two cysteines, which was expected to disrupt a zinc finger motif, abolished activity (38). Three consensus sequences have been implicated in the activity of Rho/Rac GAPs (50), and one of these resembles a sequence (158KTLAKHRVPL168) in ARD1 (identical residues underlined). Single replacement of each of the identical amino acids in p5 abolished GAP activity without preventing p3 interaction (48). Because of the importance of an arginine in the action of many GTPases (reviewed in Ref. 51), each of four arginines in the GAP region of p5 was individually mutated, thereby establishing Arg164 and Arg166 as essential for GAP activity but not for p3 association (48).

In addition to its GAP function, p5 acts as a GDP-dissociation inhibitor for p3 (45), thereby slowing activation, which along with the enhanced inactivation of p3 contributes to stabilization of the inactive state. The C-terminal 15 amino acids of p5 (those immediately preceding p3) inhibited the release of GDP but not GTP·γS from p3 (47), i.e. they influenced GDP dissociation in a manner similar to the action of the N terminus of ARF (52), which also has an effect on the specificity of nucleotide binding (53). Hydrophobicity of these sequences in ARD1 was important as shown by parallel effects of single amino acid replacements on hydrophobicity and on GDP·S dissociation (47). Roles for the N-myristoylated amphipathic terminal α-helix of ARF1 in membrane association and activation resulting from the conformational change induced by

---

2 The sequence of ARNO is virtually identical to a sequence in GenBank (accession number U7072–8) named cytohesin-2.
GTP binding had been previously postulated (54, 55). Although guanine nucleotide-dissociation inhibitory proteins for several GTPases are well known, none (as often noted) has been found for ARFs, perhaps because they, like ARD1, have an analogous intrinsic regulatory component (47). It was suggested that the mechanism of ARF interaction with GEFs might be similar to that by which guanine nucleotide exchange on Gα subunits is accelerated via positively charged amino acids in the third intracellular receptor loop (56).

When peptides and antipeptide antibodies were used to identify sites of ARF interaction in p5, it was noted that both were much less inhibitory with ARD1 than with p5 plus p3 (48). This is perhaps not surprising and is consistent with the probability that access to the interaction site is more limited when the two domains are covalently linked. Although ARD1 has provided considerable information relevant to several aspects of the regulation of ARF activity, its physiological function remains to be established.

Afterword

By far the largest part of the data on ARF properties, functions, and protein or phospholipid interactions still pertains specifically to class I ARFs. Although conditions of assays that were developed for those ARFs are, of course, not necessarily optimal for class II or class III ARFs, this is not often enough addressed experimentally. For example, the N- and C-terminal regions of ARFs, which to-for those ARFs are, of course, not necessarily optimal for class II or class I ARFs. Although conditions of assays that were developed and protein or phospholipid interactions still pertains specifically to class I ARFs. Although conditions of assays that were developed and protein or phospholipid interactions still pertains specifically to class I ARFs.

Identification and characterization of the molecules that operate and regulate intracellular vesicular transport are obviously basic to understanding the mechanisms involved. The development of ingenious in vitro assays made possible separation and purification of many of these. Although many workers continue to provide increasingly detailed information, there are numerous parts of the scaffolding, anchoring, or adaptor structures, as well as the machinery, awaiting description. In the end, all must be integrated with data concerning physical and regulatory aspects of the processes, guided by insights from structural and cell biology, to understand correctly the functional architecture of the cell and its molecular components, including ARFs.

Note Added in Proof—After this manuscript was submitted, a second group reported the crystal structure of the ARNO Sec7 domain (Cherfils, J., Antonny, B., and Chardin, P. (1998) J. Biol. Chem. 273, 22221–22226. Note Added in Proof—After this manuscript was submitted, a second group reported the crystal structure of the ARNO Sec7 domain (Cherfils, J., Antonny, B., and Chardin, P. (1998) J. Biol. Chem. 273, 22221–22226.)