Zoological Studies **34**(1): 1-9 (1995)

Zoological Studies

Review Article

ADP-ribosylation Factors: ARF Structure and Function

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ABSTRACT

Su-Chen Tsai, Ronald Adamik, Joel Moss and Martha Vaughan (1995) ADP-ribosylation factors: ARF structure and function. *Zoological Studies* **34**(1): 1-9. ADP-ribosylation factors (ARFs) are ~20-kDa guanine nucleotide-binding proteins initially discovered by their ability to activate cholera toxin ADP-ribosyltransferase activity. Subsequently they were shown to be present in the Golgi and to participate in vesicular trafficking in eukaryotic cells. ARFs are highly conserved from *Giardia* to mammals. Mammalian and *Drosophila* ARFs fall into three classes based on size, amino acid sequence, and gene structure. The ability of ARFs to activate cholera toxin requires GTP or an analogue and is promoted by phospholipids and detergents. Toxin activation is independent of the amino terminal thirteen amino acids. However, myristoylation at the amino terminus promotes association of ARFs with Golgi and other membranes. Binding to membranes appears to be enhanced by GTP and a soluble complex in a process inhibited by brefeldin A (BFA), a fungal metabolite known to disrupt Golgi. One component of the soluble complex is a phospholipid-dependent guanine nucleotide-exchange protein, which, in a purified state, is independent of BFA and enhances ARF binding of GTP. Activation of ARF by GTP binding promotes its association with Golgi and the subsequent binding of the coatomer proteins, which initiate the vesicular budding process. ARFs have recently been shown to activate phospholipase D which, through the generation of phosphatidate and its metabolites, may be responsible for some of the ARF-initiated membrane trafficking events.

Key words: Cholera toxin, ADP-ribosylation factors, Guanine nucleotide-binding proteins, Brefeldin A, Phospholipase D.

INTRODUCTION

ARF and ARF-related proteins, genes, and mRNAs

ARFs (ADP-ribosylation factors) were discovered in Gilman's laboratory (Schleifer et al. 1982, Kahn and Gilman 1984) as activators of cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$. It was in yeast, *Saccharomyces cerevisiae*, that ARFs were first implicated in Golgi function (Botstein et al. 1988, Stearns et al. 1990b). They have since been shown to participate in several types of intracellular vesicular transport. Studies with anti-ARF and anti-ARF peptide antibodies have demonstrated ARFs of similar size in all eukaryotic species, but not in

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bacteria (Kahn et al. 1988, Tsai et al. 1991b). As shown in Table 1, ARFs are highly conserved across eukaryotic species from the protozoan *Giardia* to mammals, and mammalian ARFs are also quite similar in primary sequence to ARFs cloned from *S. cerevesiae* (Sewell and Kahn 1988, Stearns et al. 1990a). Six mammalian ARFs have been identified, which can be grouped into three classes, based on size, deduced amino acid sequence, and phylogenetic analysis (Tsuchiya et al. 1991). Locations of introns in the coding region for the Class I ARFs (ARFs 1, 2, and 3) are identical (Fig. 1) and differ from those in the Class II ARFs (ARFs 4 and 5), which are identical to each other (Haun et al. 1992). All findings are consistent with the view that members of the different classes of ARFs arose through gene duplication events.

ARFs appear to be expressed in all eukaryotic tissues. During post-natal development of the rat brain, ARF3 mRNA and protein increased in parallel, whereas mRNAs for ARF2 and ARF4 decreased substantially, and those for ARF1, ARF5, and ARF6 were apparently unchanged (Tsai et al. 1991b). Amounts of mRNA for ARF1 and ARF3 in rat brain were increased by chronic administration of corticosterone and decreased by bilateral adrenalectomy (Duman et al. 1990).

To define factors that may regulate ARF3 gene expression, the human ARF3 promoter was examined in some detail (Haun et al. 1993a). It had the characteristics of a "housekeeping" gene, including the absence of a TATA or CAAT box and the presence of several GC boxes within a highly GC-rich region, as well as multiple transcription initiation sites. Sequences within 58 base pairs of the transcription initiation site were necessary for full expression, in particular, a ten-base palindrome. A DNA-binding protein, termed TLTF, was bound to an oligonucleotide containing this palindrome, and apparently also to an upstream sequence. With the promoter containing a mutated palindrome, expression was reduced dramatically, consistent with a role for this region in controlling ARF3 expression.

mRNAs for ARFs 1 (Lee C-M et al. 1992), 3 (Tsai et al. 1991c), and 4 (Mishima et al. 1992) appear to be products of alternative polyadenylation. The same two sites of polyadenylation and polyadenylation signals were found in the rat and human ARF1 gene products (Lee C-M et al. 1992). ARF3 mRNAs of similar size were observed in a variety of mammalian species (Bobak et al. 1989), consistent with the conservation of polyadenylation sites (Fig. 1). In the case of ARF4, testis-specific expression of short forms of mRNA was observed in several species (Mishima et al. 1992). During mouse development, appearance of the short form coincided with late spermatogenesis, whereas other ARF mRNAs had the same size as those detected in somatic cells. Why only ARF4 mRNA is synthesized as a short form during spermatogenesis is an intriguing question.

ARFs have been identified in all eukaryotic species investigated and appear to be highly conserved. *Giardia lamblia* is a protozoan intestinal parasite that is believed to be among the most

Table 1. Comparison of ARF nucleotide and deduced amino acid sequences

	hARF1	bARF1	bARF2	hARF3	hARF4	hARF5	hARF6	yARF1	gARF
hARF1		100	96	96	80	80	68	77	70
bARF1	91		96	96	80	80	68	77	70
bARF2	79	80		95	80	80	69	77	70
hARF3	84	84	80		79	79	68	76	69
hARF4	67	68	68	71		90	64	72	69
hARF5	75	73	71	73	77		64	69	69
hARF6	68	69	64	66	60	65		65	63
yARF1	64	66	66	65	67	64	60		67
gARF	65	67	62	64	61	66	62	62	

Percentage identity of nucleotide sequences (coding regions) is below the diagonal, percentage identity of deduced amino acid sequences above. hARF1, human ARF1 (Bobak et al. 1989); bARF2, bovine ARF2 (Price et al. 1988); hARF3, human ARF3 (Bobak et al. 1989); hARF4, human ARF4 (Monaco et al. 1990); hARF5, human ARF5 (Tsuchiya et al. 1991); hARF6, human ARF6 (Tsuchiya et al. 1991); yARF1, yeast ARF1 (Sewell and Kahn 1988); gARF, Giardia ARF from isolate WB. Data from Murtagh et al. (1992).

ancient existing eukaryotes. A *Giardia* ARF (gARF) gene had a 573-base open reading frame encoding a 191-amino acid protein that is 63-70% identical to known mammalian and yeast ARFs (Murtagh et al. 1992). The gARF, expressed in *E. coli* as a fusion protein, activated cholera toxin, and when expressed in *S. cerevisiae*, was capable of rescuing the lethal *arf1*$^-$*/arf2*$^-$ double mutant (Lee F-JS et al. 1992). Growth was, however, considerably slower than it was when yARF1 was used for complementation, consistent with the likelihood that the gene products are not identical in function. The lethal mutant was also rescued by expression of hARF1, hARF4, hARF5, or hARF6 (Stearns et al. 1990a, Kahn et al. 1991, Lee F-JS et al. 1992).

Mammalian ARFs 1 and 3 (class I) have been implicated in several pathways or processes of intracellular membrane trafficking, but it has been difficult to obtain clues to the physiological actions of ARFs 4, 5, and 6. The *Drosophila melanogaster* counterparts of these ARFs were sought in the hope that they might be easier to study successfully in this organism, which has many experimental advantages, and about which there is so much genetic and developmental information. ARFs that appear to be examples of each of the three mammalian classes have now been identified in *D. melanogaster* (Murtagh et al. 1993, Lee F-JS et al. 1994). The three ARFs were more similar to those of the same classes in other species than to each other, were located on different chromosomes, and were differently expressed in heads vs. legs vs. bodies, consistent with the conclusion that they represent independent entities.

ARF STRUCTURE AND FUNCTION

Studies in several laboratories in association with R.A. Kahn have led to the proposal that the amino terminus of ARF is critical for its effectiveness in cholera toxin activation (Kahn et al. 1992), endocytosis (Lenhard et al. 1992), and vesicular transport between endoplasmic reticulum and Golgi (Balch et al. 1992). A role for the amino terminus was postulated based on the ability of a hexadecapeptide representing the amino terminal sequence (without methionine) of ARF1 to interfere with these processes. To investigate this question further, ARF1 amino terminal deletion mutants were synthesized in *E. coli* and assayed for their ability to activate cholera toxin in a GTP-dependent manner (Hong et al. 1994). These included a mutant of ARF1 lacking the first thirteen amino acids and a mutant in which the first fourteen amino acids of ARF1 were replaced with the first seven amino acids of the cAMP-dependent protein kinase catalytic subunit. Both mutants caused GTP-dependent activation of toxin similar to that of rARF1. These studies are consistent with the conclusion that the amino terminus of rARF1 is not critical for its action as a GTP-dependent activator of cholera toxin. It appears then that the ability of the ARF1 peptide to inhibit in other studies may not, in fact, reflect a specific effect on the ARF-toxin interaction, since the amino terminus is not required for toxin activation. It seems possible that at least some of the effects attributed to the ability of peptide to mimic ARF may have another explanation.

A somewhat surprising result of these experiments was the observation that the recombinant

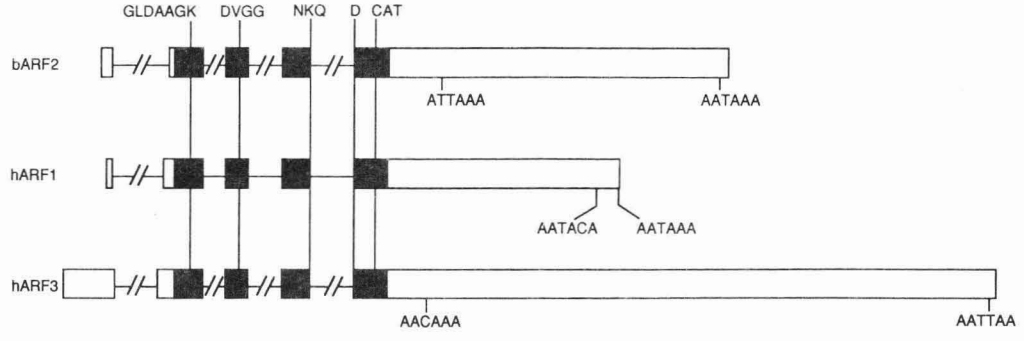

Fig. 1. Structures of class I ARF genes. Exons are represented by *boxes* and introns by *horizontal lines*. *Shaded boxes* represent the coding regions, and *open boxes* represent 5′- and 3′-UTRs. Amino acid consensus sequences for guanine nucleotide-binding and GTP hydrolysis domains are noted above the bARF2 gene and are represented in the hARF1 and hARF3 genes as *vertical lines*. Sequences of polyadenylation signals are below each gene. Genes are drawn to scale with the hARF1 gene as the size reference. Data for hARF1, bARF2, and hARF3 genes are in Lee C-M et al. 1992, Serventi et al. 1993, and Tsai et al. 1991c, respectively. Diagram from Serventi et al. 1993.

mutant ARF proteins, as isolated, were fully active, i.e., their activity was unaffected by added GTP (Hong et al. 1994). Recombinant ARF proteins are almost always isolated in a GTP-independent state with GTP bound. The reason for this has been unclear, since ARFs apparently lack intrinsic GTPase activity, and in *E. coli*, as in eukaryotic cells, the GTP concentration is higher than that of GDP. ARF6 synthesized as a fusion protein with maltose-binding protein was active as isolated, and most of its bound nucleotide was identified as GTP (Welsh et al. 1994). GTP-dependence was restored to the mutant ARFs by treatment with 7M urea followed by renaturation (Hong et al. 1994). These mutants may be useful in defining the role of the ARF amino-terminus in protection of bound GTP from hydrolysis by extrinsic proteins, and/or its contribution to an overall active or inactive conformation.

Effects of phospholipids on ARF activity

Kahn and Gilman (1984) initially reported the importance of detergent for ARF stability and activity. High affinity GTP binding by sARFII (later identified as ARF3) was observed only in the presence of dimyristoylphosphatidylcholine and cholate; these lipids similarly lowered the apparent EC50 for GTP in the ARF-activated cholera toxin assay (Bobak et al. 1990). Differences in the effects of phospholipids on activities of recombinant ARFs from the three classes were later shown (Price et al. 1992).

The cholera toxin-catalyzed ADP-ribosylation reaction displays negative cooperativity, i.e., binding of one substrate decreases affinity for the other (Osborne et al. 1985). Noda et al. (1990) had shown that ARF decreased the apparent K_m for both substrates without altering V_{max}, and addition of SDS caused a further decrease in K_m values for both ADP-ribose donor and acceptor along with an increase in V_{max}. Cardiolipin and mixtures of sodium cholate with phosphatidylinositol, or phosphatidylserine also decreased the K_m for NAD in the presence of ARF (Murayama et al. 1993). The influence of certain phospholipid/detergent mixtures on the formation of stable complexes of ARF and toxin, that differ in substrate specificity from mixtures of ARF and toxin that are not associated in this way, has been described (Tsai et al. 1991a). More recently, the importance of phosphatidylserine in the exchange of ARF-bound GDP for GTP, catalyzed by a guanine nucleotide-exchange protein, was reported (Tsai et al. 1994), and the critical composition of the phospholipid substrate that permitted demonstration of ARF activation of phospholipase D was pointed out by Brown et al. (1993).

Although ARFs are apparently predominantly soluble proteins, they associate with membranes and phospholipids in a GTP-dependent manner (Kahn 1991, Walker et al. 1992). In contrast to other ~20-kDa guanine nucleotide-binding proteins, ARFs are myristoylated at their amino termini (Kahn et al. 1988, Kunz et al. 1993, Haun et al. 1993b). Using a bacterial expression system that permitted coexpression of the yeast N-myristoyltransferase and the human ARF5 genes, recombinant myristoylated and non-myristoylated ARF5 were prepared (Haun et al. 1993b). Myristoylated and non-myristoylated ARF5 exhibited similar activities as measured by guanine nucleotide binding and activation of cholera toxin. Myristoylated ARF5, however, demonstrated a temperature and time-dependent association with Golgi, whereas non-myristoylated ARF5, did not bind to Golgi. These studies were consistent with the view that myristoylation is necessary but not sufficient for membrane association, and is not necessary for ARF activation of cholera toxin.

ARF FUNCTION IN INTRACELLULAR MEMBRANE TRANSPORT

Most of the information on ARF function in cells is related to vesicular transport in the Golgi (Serafini et al. 1991), probably involving class I ARFs. ARFs have also been implicated in endoplasmic reticulum to Golgi transport (Balch et al. 1992, Dascher and Balch 1994), endocytosis (Lenhard et al. 1992), and nuclear membrane assembly (Boman et al. 1992). During the last year or two, exciting work in several laboratories (notably those of Rothman, Orci, Schekman, and Balch) has been moving at an accelerating pace toward the gratifying demonstration that proteins and processes that operate in vesicle fusion with a target membrane are basically analogous in different types of cells and at different loci within cells (Steinhardt et al. 1994). Some of the most extensive, detailed and successful studies of vesicular transport, including vesicle formation, targeting and fusion have involved intra-Golgi transport utilizing an in vitro system initially devised in Rothman's laboratory (Osterman et al. 1993). In this system, the roles of ARF and coatomer in vesicle formation were defined, the NEM-sensitive fusion (NSF) protein was identified, and other re-

quirements for vesicle fusion with target membranes were worked out (Osterman et al. 1993). It is believed that to initiate intracellular membrane vesicle formation, binding of ARF·GTP is required. In the cell, ARF·GDP is cytosolic. Interaction with a specific guanine nucleotide-exchange protein (GEP) accelerates exchange of GDP for GTP (Donaldson et al. 1992, Helms and Rothman 1992, Randazzo et al. 1993, Tsai et al. 1994), producing ARF·GTP, which can bind to phospholipids or membranes. In cells, it binds presumably to a specific membrane region at which vesicle budding will occur. Whether this involves a specific ARF "receptor" protein or some other means of defining the interaction site has not been established.

Incubation of post-nuclear brain supernatant with GTP*y*S increased the recovery of ARF activity in sucrose density gradient fractions containing Golgi. This association of ARFs with Golgi was dependent on an ATP-regenerating system and temperature. Based on immunoreactivity on Western blots and assay of ARF activity, ARFs 1, 3, and 5 behaved independently and selectively in their GTP-dependent association with Golgi in vitro. Although ARF3 was much more abundant than ARF1 in rat brain (Tsai et al. 1991b), more ARF1 than ARF3 was bound to Golgi, whether mixed ARFs in cytosol or purified ARF1 and ARF3 (Tsai et al. 1988) were used (Tsai et al. 1992). It was noted that the ratio of ARF activity to galactosyl transferase activity (a Golgi compartment marker enzyme) differed greatly in different fractions, presumably because specific ARFs associate with membranes (Golgi or other) different from those that contain the transferase.

Brefeldin A, a fungal lipid metabolite, disrupts Golgi function and inhibits the binding of soluble high molecular weight proteins referred to as COPs, which are components of the protein complexes identified as coatomers (Serafini et al. 1991, Osterman et al. 1993), to Golgi fractions (Klausner et al. 1992). Effects of brefeldin A on binding of ARFs 1, 3, and 5 to a rat brain Golgi fraction in the presence of an ATP-regenerating system, and a fraction of soluble, high molecular weight accessory proteins, presumably containing the coatomer protein complexes were investigated. Binding of all was dependent on GTP*y*S and increased by an ATP-regenerating system. Binding of ARFs 1 and 3 but not ARF5 was increased by the accessory protein fraction (Tsai et al. 1993). Brefeldin A inhibited the accessory protein-dependent, but not the independent, binding of ARFs 1 and 3. It had no effect on the increment produced by the ATP-regenerating system. B36, an inactive derivative of brefeldin A, did not affect the accessory protein-dependent binding. Binding of ARF5, which was accessory protein-independent, was not affected by brefeldin A. Recombinant myristoylated ARF5 and ARF5 partially purified from rat brain behaved similarly to ARF5 in cytosol (Tsai et al. 1993). It appears that some, possibly large, fraction of ARF binding, although GTP-dependent is "nonspecific" (Helms et al. 1993), perhaps analogous to the GTP-dependent binding of ARF to phospholipids (Kahn 1991, Walker et al. 1992). BFA inhibition of binding of ARF1 and ARF3 appears to define one relatively specific component, i.e., that which is dependent on something(s) in the accessory protein fraction.

Exchange of GDP bound to ARF1 for GTP was shown by other investigators to be enhanced by Golgi membranes in a brefeldin A-sensitive reaction, leading to the conclusion that a guanine nucleotide-exchange protein (GEP) was the brefeldin A target (Donaldson et al. 1992, Helms and Rothman 1992, Randazzo et al. 1993). To establish the site of brefeldin A activity and to characterize the guanine nucleotide-exchange reaction, a soluble GEP was partially purified from bovine brain (Tsai et al. 1994). GEP-dependent exchange of nucleotides on ARFs 1 and 3, assayed as increased ARF activity in the toxin assay or increased [^{35}S]GTP*y*S binding, was markedly enhanced by phosphatidylserine, although cardiolipin was, in fact, more effective than phosphatidylserine in enhancing ARF-stimulated cholera toxin activity. GEP increased the exchange rate, but, as expected, did not affect the affinity of ARF for GTP. The crude GEP behaved as a molecule of ~700 kDa, whereas after partial purification, it behaved on Ultrogel AcA 54 chromatography as a protein of ~60 kDa. With purification, GEP activity became brefeldin A insensitive, consistent with the conclusion that the exchange protein is not itself the BFA target (Tsai et al. 1994). It appears that the loss of BFA inhibition with purification is related to separation of a ~60-kDa GEP molecule from another protein(s) that is sensitive to BFA, with which it was initially associated in the larger complex. That complex may include other regulatory proteins and/or proteins that interact with ARF along with coatomer to initiate coatomer binding and budding.

Whether ARF·GTP association with the membrane leads directly or indirectly to rearrangement (or formation) of membrane phospholipid domains is also unknown. Subsequent binding of large

protein complexes, termed coatomers, then results in bud formation. Requirements for each of these steps have been rigorously defined in Rothman's laboratory, where it was shown that ARF and coatomers are the only cytosolic proteins needed for Golgi vesicle budding (Osterman et al. 1993). When that process was carried out with the purified proteins, especially prepared membranes and GTP, release of vesicles, i.e., membrane bilayer fusion to close off the base of the bud, required only the addition of fatty acyl CoA. These vesicles were then capable of fusing with Golgi acceptor membranes in the presence of cytosol (which provided components of the fusion machinery), ATP and an ATP-regenerating system (Osterman et al. 1993). Fusion did not occur when vesicles had been prepared with GTPγS or with a mutant ARF that does not hydrolyze GTP, consistent with the earlier conclusion that uncoating of the vesicle is a prerequisite for fusion. Uncoating means release of coatomers, which requires prior release of ARF·GDP. It is presumed that conversion of ARF·GTP to ARF·GDP occurs when ARF interacts with a GTPase-activating protein (GAP) in the target membrane. Cycling of ARF between the GDP-bound cytosolic and GTP-bound membrane-associated forms is shown schematically in Fig. 2.

The relatively recent reports of activation of phospholipase D by ARF·GTP (Brown et al. 1993, Cockcroft et al. 1994) permit the speculation that this enzyme could be an ARF·GAP as well as an effector, although there is no evidence for this. There is evidence that effectors can function as GAPs for certain other GTP-binding proteins. As has been suggested (Brown et al. 1993, Cockcroft et al. 1994), phosphatidic acid produced by phospholipase D action at the target site, could serve as a fusogen to complete the transport step. The role of lipid bilayer asymmetry in membrane fusion has been extensively studied (Devaux 1991). Based on experiments with model membranes, it has been suggested that fusion might be regulated by "local enzymatic generation of fusogenic lipids such as diglycerides or phosphatidic acid" (Eastman et al. 1992). There is evidence of several kinds that specific membrane pathways of vesicular transport involve additionally in their operation or regulation other "small" ~20-kDa GTP-binding proteins (e.g., rabs) as well as heterotrimeric GTP-binding proteins (Bomsel and Mostov 1992). Although only ARF1 and ARF3 (class I ARFs) have been directly implicated in the Golgi membrane systems, it seems likely that the other ARFs will play analogous roles in different intracellular membranes, i.e., as the sources of signals that trigger coating and uncoating of vesicles, which are, therefore, required for vesicle formation and successful fusion.

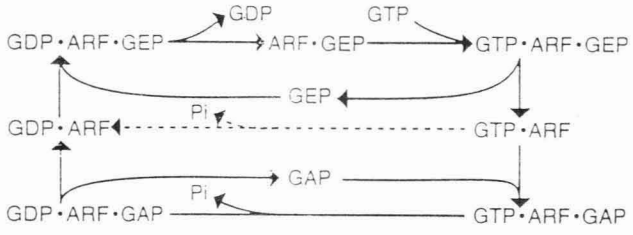

Fig. 2. Regulation of ARF Activity by GEP and GAP. Postulated roles are indicated for GEP (guanine nucleotide exchange protein) and GAP (GTPase-activating protein). Interrupted arrow (---) indicates the absence of detectable GTP hydrolysis by natural ARF (Kahn and Gilman 1986) or by a recombinant ARF, synthesized in *E. coli* and isolated with GTP bound (Welsh et al. 1994).

Acknowledgements: We thank Mrs. Barbara Mihalko for expert secretarial assistance and the many members of the Laboratory of Cellular Metabolism, past and present, who are responsible for much of the work discussed here.

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腺苷二磷酸核醣基化因子：ARF之構造與功能

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腺苷二磷酸核醣基化因子(ARFs)係分子量約20 KDa的鳥嘌呤核苷酸結合蛋白。最初由於其具有活化霍亂毒素腺苷二磷酸核醣基化轉移酶的能力而被發現，爾後發現此因子亦存在於高爾基體，並參與其眞核細胞囊胞的運送。從賈弟蟲(Giardia)到哺乳類，ARFs具有高度穩定性。依胺基酸序列、分子大小及基因構造，哺乳類和果蠅的ARFs可分爲三類。ARFs需要GTP(鳥糞嘌呤核苷三磷酸鹽)或類似物以活化霍亂毒素，而磷脂與清潔劑可促進其作用。霍亂毒素之活化與ARFs的N-端十三個胺基酸無關，然而ARFs之N端十四烷酸化可增強ARFs與高爾基體和其它膜之結合。GTP和形成的水溶性複合體可能會促進ARFs與膜之結合。水溶性複合體的形成過程被brefeldin A (BFA)所抑制，此BFA是一種會破壞高爾基體的眞菌代謝物。水溶性複合體中有一種成分即與磷脂有關的鳥嘌呤核苷酸交換蛋白質。此種經純化的蛋白質與BFA無關，但可促進ARFs與GTP的結合，進而與高爾基體結合，最後和Coatomer蛋白結合引起囊胞發芽。最近研究結果顯示ARFs可活化磷脂酶D (phospholipase D)，其藉由磷脂酸鹽(Phosphatidate)與其代謝物的產生，可能和ARFs啟始膜(囊胞)之運送功能有關。

關鍵詞：霍亂毒素，腺苷二磷酸核醣基化因子，鳥嘌呤核苷酸結合蛋白，Brefeldin A，磷脂酶D。

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