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Guanine Nucleotide-Exchange Factors for Arf GTPases: Their Diverse Functions in Membrane Traffic

Hye-Won Shin* **and Kazuhisa Nakayama†**
Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501

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Small GTPases of the Arf family, by cycling between GDP-bound inactive and GTP-bound active states, play a crucial role not only in the regulation of membrane traffic and dynamics but also in rearrangement of actin cytoskeleton. The exchange of GDP for GTP on Arf is catalyzed by a family of guanine nucleotide–exchange factors (GEFs) containing a Sec7 domain. The Sec7 domain is a target of brefeldin A, which inhibits various trafficking processes and induces organelle disintegration. During the past few years, significant progress has been made in elucidating the structure and catalytic mechanism of the Sec7 domains and physiological functions of the Sec7 domain–containing Arf-GEFs. Here we review the structures and functions of Arf-GEFs by focusing on the regulation of membrane traffic.

Key words: Arf, brefeldin A, Golgi apparatus, membrane traffic, Sec7 domain.

Abbreviations: Arf, ADP-ribosylation factor; GEF, guanine nucleotide-exchange factor; GAP, GTPase-activating protein; BFA, brefeldin A; TGN, *trans*-Golgi network; PKA, protein kinase A.

The small GTPases of the ADP-ribosylation factor (Arf) family function in eukaryotic cells to regulate membrane traffic and dynamics. There are six Arfs (Arf1–Arf6) in mammals and three (Arf1p–Arf3p) in the yeast *Saccharomyces cerevisiae*. Mammalian Arfs are structurally divided into three classes: class I, Arf1–Arf3; class II, Arf4 and Arf5; and class III, Arf6 (*1*, *2*). Of these, Arf1, which regulates various aspects of membrane traffic, and Arf6, which regulates endocytic and recycling processes and cytoskeletal remodeling, have been extensively studied (*3*, *4*). Eukaryotic cells also have several Arl (for Arf-like) proteins with diverse functions (*2*).

Like other GTPases, Arf switches between a GDP-bound inactive state and a GTP-bound active state, in which it interacts with a variety of effector proteins (Fig. 1A). The exchange of GDP for GTP on Arf is catalyzed by guanine nucleotide-exchange factors (GEFs). On the other hand, the bound GTP is hydrolyzed to generate GDP with the aid of GTPase-activating proteins (GAPs) (*5*–*7*). The GDP-bound form of Arf1 is cytosolic or associates loosely with membranes. In contrast, the GTP-bound form associates tightly with organelle membranes through the myristoyl moiety attached at its N-terminus and the N-terminal α-helical region. It recruits coat proteins, such as the COPI complex, the AP-1 clathrin adaptor complex and the GGA proteins, to trigger budding of coated carrier vesicles (Fig. 1B). Thus, activation of Arf by Arf-GEFs is a critical regulatory step for vesicular trafficking. All Arf-GEFs identified so far possess an ~200–amino acid Sec7 domain (Fig. 2) that is alone responsible for the GEF activity (*8*). The Sec7 domain is now known to be a target of the fungal metabolite brefeldin A (BFA). Although, in studies of membrane traffic, BFA is the most frequently used laboratory tool that blocks various trafficking processes and causes disintegration of various organelles including the Golgi apparatus and endosomes (*9*–*11*), its target had long been unknown. In 1992, three groups independently demonstrated the existence of BFA-sensitive guanine nucleotide exchange activities toward Arfs on Golgi membranes (*12*–*14*). In addition, our previous study suggested the existence of at least two BFA-sensitive Arf-GEFs in the cell; one is involved in membrane recruitment of COPI at the *cis*-Golgi and the other in recruitment of AP-1 at the *trans*-Golgi network (TGN) (*15*).

The Sec7 family proteins

SEC7 was identified by Schekman and colleagues as one of the genes implicated in the process of protein transport in the yeast *S. cerevisiae*, and its gene product was later found to associate with the Golgi apparatus and be required for membrane traffic through and from this organelle (*16*, *17*). The first hint about the relationship between Arf and the Sec7 domain was presented about the middle of 1996; Franzusoff and colleagues reported that overexpression of not only yeast Arf1p and Arf2p but also human Arf4 rescued the yeast *sec7* mutant (*18*). At the end of the same year, two groups independently showed that Sec7 domain–containing proteins are Arf-GEFs. Moss, Vaughan and colleagues purified a 200-kDa BFA-sensitive Arf-GEF from bovine brain cytosol, which is now called BIG1, and showed that sequences of tryptic peptides from this protein had significant similarity to those in yeast Sec7p (*19*). Jackson, Chardin and colleagues identified Gea1p, which possesses a region homologous to Sec7p, as a multi-copy suppressor of the yeast growth defects conferred by dominant-negative Arf2p and subsequently showed that yeast Gea1p and human ARNO, a member of the CYT subfamily (see

*A research fellow of the 21st Century COE Program.
†To whom correspondence should be addressed. Tel: +81-75-753-4527, Fax: +81-75-753-4557, E-mail: kazunaka@pharm.kyoto-u.ac.jp

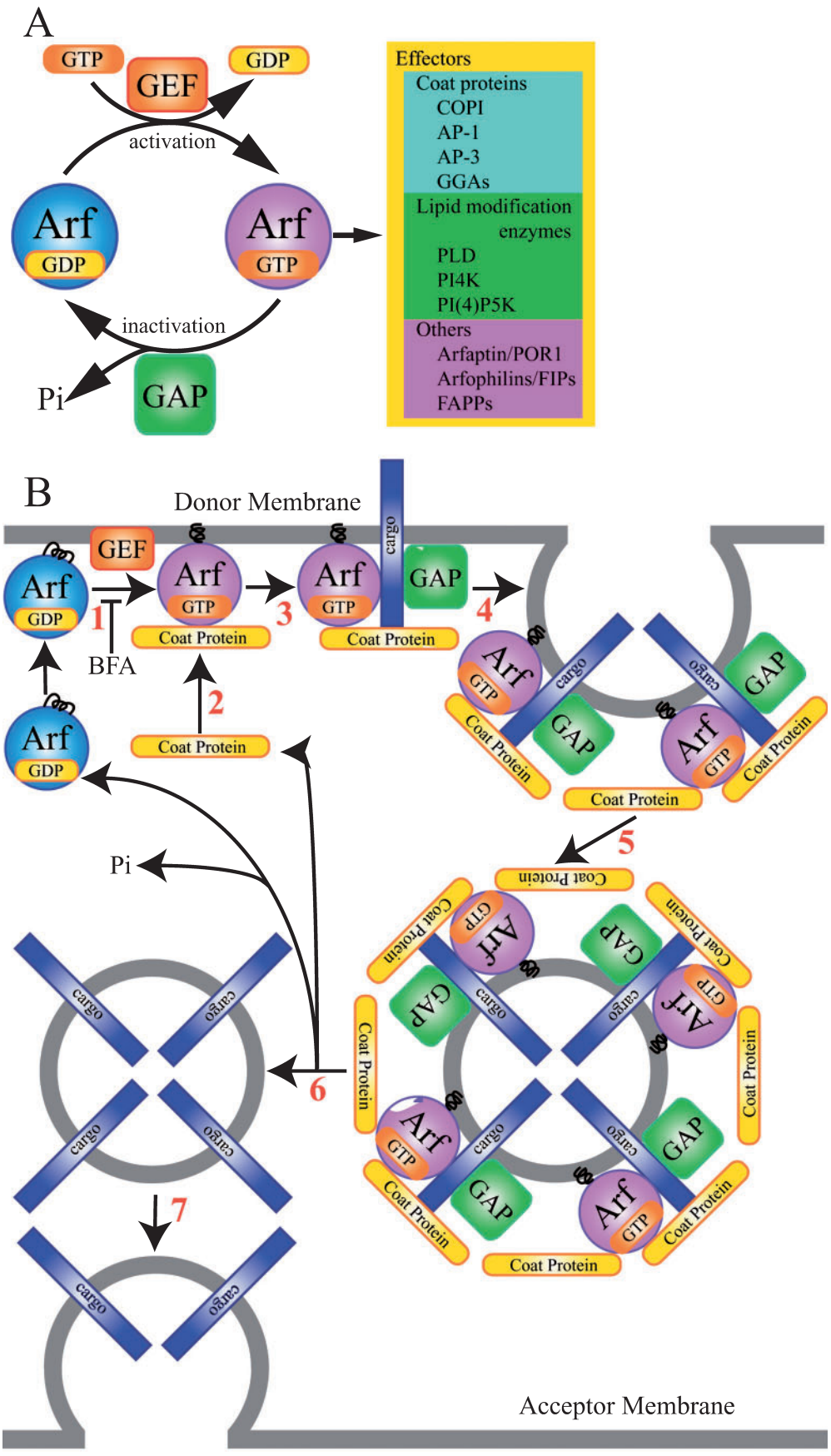

Fig. 1. **GDP-GTP cycle of Arf and its function in coated vesicle formation.** (A) General regulation of Arf activation and inactivation. A GDP-bound, inactive form of Arf is converted to a GTP-bound, active form through GDP-GTP exchange catalyzed by a GEF. The GTP-bound Arf is able to interact with a wide variety of effectors, such as coat proteins and lipid kinases. The GTP molecule bound to Arf is then hydrolyzed to GDP with the aid of a GAP. (B) Regulation of coated vesicle formation by Arf. (1) Through guanine nucleotide exchange catalyzed by a GEF on a donor membrane, a GDP-bound form of Arf in the cytosol or loosely associated with the membrane is converted to a GTP-bound form, which becomes tightly associated with the membrane. The exchange reaction is blocked by BFA. (2) Arf·GTP then recruits coat proteins from the cytosol. (3) Arf and the coat together trap transmembrane cargo at the donor membrane. Recognition of a sorting signal within the cytoplasmic domain of the cargo by the coat is critical for this step. A GAP is also recruited to the membrane, but its activity might be inhibited due to the cargo (see Ref. *6*). (4) The GAP inhibition might shift the equilibrium to drive coat polymerization, and in consequence, the membrane patch deforms into a bud. (5) The vesicle is then pinched off from the donor membrane. (6) As the membrane curves, the GAP activity might increase, leading to GTP hydrolysis and dissociation of the resulting Arf•GDP, the coat and the GAP itself (see Ref. *63*). (7) The uncoated vesicle is now competent to fuse with an appropriate acceptor membrane.

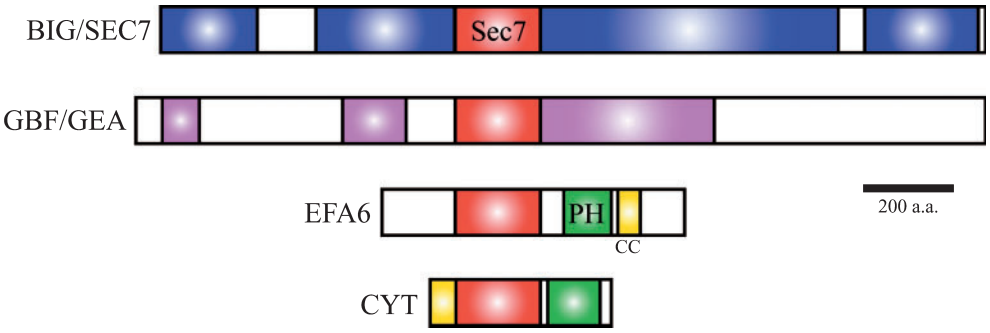

Fig. 2. **Schematic representation of structures of the Arf-GEF subfamilies.** The Sec7 domains are highlighted in red, and the pleckstrin homology (PH) domains and coiled-coil (CC) regions are shown in green and yellow, respectively. Regions conserved within the BIG/SEC7 and BIG/GEA subfamilies are shown in blue and magenta, respectively.

below), promote guanine-nucleotide exchange on Arfs (*20*, *21*). Since then, Arf-GEFs have been extensively studied.

On the other hand, in 1994, Shevell *et al.* reported that mutations in the *EMB30* [also known as *GNOM* (*22*)] gene locus in *Arabidopsis thaliana* caused defects in apical-basolateral pattern formation and its product has similarity to yeast Sec7p (*23*). They referred to the Sec7-homologous region as the Sec7 domain. Notably, they found that one of the mutant alleles, *emb30-1*, has a mutation in the Sec7 domain that alters a conserved Glu residue to Lys (E658K) (*23*). The critical role of the Glu residue, the so-called Glu finger, in the catalytic activity was later revealed by structural studies (*24–26*) (see below).

In the genome databases, there are 15 Sec7 domain–containing proteins in humans, 8 in *A. thaliana*, 5 each in *Drosophila melanogaster*, *Caenorhabditis elegans* and *S. cerevisiae* (*8*). Recently, Cox *et al.* have performed a phylogenic analysis and divided these proteins into seven main subfamilies, designated BIG/SEC7, GBF/GEA, CYT, EFA6, BRAG, SYT1 and SYT2 (Fig. 2) (*8*). The CYT, EFA6 and BRAG subfamilies are specific for animals, and the SYT1 and SYT2 subfamilies for fungi (*8*). Two parasite bacteria have proteins with Sec7 domains that may have been acquired by horizontal gene transfer of a eukaryotic gene (*8*, *27*). Among the seven subfamilies, members of the former four subfamilies are relatively well characterized. Members of the BIG/SEC7 and GBF/GEA subfamilies consist of >1,400 amino acids and are often referred to as large or high-molecular-weight Arf-GEFs, while the CYT members consist of ~400 amino acids and are referred to as small or low-molecular-weight Arf-GEFs. The EFA6 members are intermediate in size. Essentially, regions outside the Sec7 domain have no significant similarity across the subfamilies, except that the CYT and EFA6 members have a PH domain and a coiled-coil region in common (Fig. 2). The PH domains of the CYT and EFA6 members are responsible for membrane recruitment of these proteins by binding to phosphoinositides, especially PtdIns(*4*,*5*)P_2 and PtdIns(*3*,*4*,*5*)P_3 (*7*, *28*, *29*).

Hereafter, we will focus on recent advances in understanding the functions of large Arf-GEFs, namely, the BIG/SEC7 and GBF/GEA members, because they regulate anterograde and retrograde trafficking from and through the Golgi apparatus by activating Arfs and recruiting coat proteins. For the CYT and EFA6 members that are involved in regulation of endocytic and recycling processes and remodeling of actin cytoskeleton through activating mainly Arf6, the reader is directed towards recent excellent reviews (*3–5*, *7*).

Subcellular localization and functions of GBF/GEA members

The GBF/GEA subfamily includes *S. cerevisiae* Gea1p and Gea2p, mammalian GBF1 and *A. thaliana* GNOM/EMB30. In *S. cerevisiae*, Gea1p and its homologue Gea2p appear to be functionally redundant, albeit not completely, since yeast cells lacking either gene showed no apparent growth defect, whereas the double null mutant was lethal (*20*, *30*). Temperature-sensitive *gea1* mutants in a *gea2*Δ strain appeared defective in anterograde and retrograde trafficking steps between the ER and Golgi and intra-Golgi trafficking under restrictive conditions (*20*, *30*, *31*). In addition, the mutants showed defects in actin cytoskeleton organization, and *GEA1* and *GEA2* showed genetic interactions with components of actin cytoskeleton and Arf3p, which is a counterpart of mammalian Arf6 (*32*). These data suggest that yeast Gea1p and Gea2p have comparable functions to mammalian CYT and EFA6 members.

GBF1 was first isolated by Melançon and colleagues as a factor whose overexpression conferred BFA resistance on mammalian culture cells (*33*). Golgi-enriched membrane fractions from cells with exogenous GBF1 expression showed guanine-nucleotide exchange activity toward class I Arfs (Arf1 and Arf3) in a BFA-resistant manner, whereas those from control cells showed a BFA-sensitive activity (*33*). Curiously, partially purified recombinant GBF1, however, did not show significant activity toward class I Arfs *in vitro* but catalyzed guanine-nucleotide exchange on Arf5 (class II) in a BFA-resistant manner (*33*). In contrast, our *in vivo* analysis suggested that GBF1 is active toward both class I and class II Arfs (*34*). Immunofluorescence analyses showed that GBF1 colocalizes well with *cis*-Golgi markers, such as p115 and ERGIC-53, and with β-COP, a subunit of the COPI complex (*34–36*). Furthermore, on peripheral punctate structures, GBF1 also colocalizes with Sec31, a COPII subunit, suggesting its association with the ER exit sites and/or structures apposed to the exit sites (*35*). Immunoelectron microscopic analyses indicated that GBF1 associates primarily with vesicular and tubular elements apposed to the *cis*-face of Golgi stacks, while a minor fraction associates with the Golgi stacks (*33*, *34*).

Several lines of evidence suggest that GBF1 is involved in membrane recruitment of the COPI complex through

activating Arfs and collaborating with Rab1. First, GBF1 colocalizes with β-COP not only in the Golgi region but also on peripheral punctate structures that represent pre-Golgi intermediates/ER-Golgi intermediate compartment (*35*). Second, overexpression of GBF1 is able to block BFA-induced redistribution of Arfs and COPI (*34*). Third, like in BFA-treated cells, β-COP is redistributed into the cytoplasm in cells expressing an E794K mutant of GBF1 (*35*), which corresponds to the EMB30(E658K) mutant (*23*). Finally, expression of GBF1 or Arf1 rescues COPI dissociation induced by dominant negative Rab1 (*37*).

Recently, plant GNOM/EMB30 was unexpectedly found to associate with endosomes and suggested to be required for recycling of auxin transport components (*38*).

It remains poorly understood how the association of the GBF/GEA members with Golgi membranes is regulated, although the association may be important for determining where and when Arf is activated and in consequence COPI-coated vesicles are formed (*34*). Peyroche, Jackson and colleagues have recently reported that Gea1p and Gea2p interact with an integral Golgi membrane protein, Gmh1p, through a region downstream of the Sec7 domain conserved in the GBF/GEA subfamily (*39*). Furthermore, they showed that its human counterpart, hGMH1, is localized to the *cis*-Golgi and, upon BFA treatment, redistributed to punctate structures characteristic of pre-Golgi intermediates. However, membrane association of Gea2p is marginally affected in *gmh1*Δ cells. Thus, it seems unlikely that Gmh1p is a Golgi receptor for the Gea proteins. The same group has also reported that Gea2p interacts with a P-type ATPase Drs2p at the Golgi (*40*). However, it is currently not clear whether the interaction is physiologically implicated in the Golgi localization of Gea2p. On the other hand, GBF1 was shown to interact with a Golgi-membrane tethering protein p115, yet the interaction is not required for targeting of GBF1 or p115 to membranes (*41*).

Subcellular localization and functions of the BIG/SEC7 members

The BIG/SEC7 subfamily includes *S. cerevisiae* Sec7p, mammalian BIG1 and BIG2, and *A. thaliana* BIG1-BIG5 (*8*). Analyses using temperature-sensitive *sec7* mutants suggested that Sec7p regulates intra-Golgi and post-Golgi traffic (*17*). Notably, although wild-type *S. cerevisiae* has no apparent Golgi stacks, *sec7* mutants accumulate stacks at restrictive temperature that resemble those found in mammalian cells (*42*). This phenotype is also found in *arf1*Δ*arf2*Δ cells (*43*). These observations suggest a block in trafficking from the Golgi under these conditions. Immunoelectron microscopic analysis revealed that in *Pichia pastoris*, which is also a budding yeast but contains ordered Golgi stacks, Sec7p is concentrated at the *trans*-side of the Golgi stacks (*44*). Furthermore, the *SEC7* gene is unable to rescue a *gea1*Δ*gea2*Δ strain (*7*, *31*), indicating that Sec7p is functionally different from Gea1p and Gea2p.

At light microscopic levels, both BIG1 and BIG2 are found in the perinuclear Golgi region (*36*, *45–47*), and BIG1 appears to localize mainly on the *trans* side of the Golgi in contrast to the *cis* localization of GBF1 (*36*). An Immunoelectron microscopic study showed that BIG2 concentrates in the TGN (*48*). At this compartment, BIG2 is likely to be responsible for membrane recruitment of the AP-1 clathrin adaptor complex through activating Arf, because BIG2 overexpression blocks BFA-induced redistribution of Arf1 and AP-1 but not that of COPI (*46*), and because catalytically inactive BIG2 [BIG2(E738K)] causes redistribution of AP-1 but not of COPI (*49*). Most recently, we have obtained evidence that BIG2 associates with the recycling endosomes as well as the TGN and is implicated in the endosomal integrity through activating class I Arfs; expression of BIG2(E738K) induces membrane tubules from the recycling endosomes (*50*).

Certain findings suggest that BIG1 and BIG2 form a complex in mammalian cells (*19*, *45*). However, the cellular functions of BIG1 and BIG2 do not appear to completely overlap. For example, it has been reported that BIG1 shows GEF activity toward class I and class II Arfs but not toward Arf6 (*51*), whereas BIG2 is equally effective on all classes of Arfs *in vitro* (*52*), although it appears to be specific for class I Arfs *in vivo* (*50*). Another difference between BIG1 and BIG2 is the unexpected finding that a large fraction of BIG1, but not BIG2, was accumulated in the nucleus when cells were cultured under serum-starved conditions (*53*). Furthermore, the nuclear accumulation was enhanced by incubation of the serum-starved cells with the immunodepressant drug FK506. These data suggest the participation of BIG1 in both Golgi and nuclear functions, although future studies will be required to address the physiological relevance of the nuclear accumulation. The functional importance of BIG2 is underscored by the finding that mutations in the BIG2 gene are associated with a human hereditary disorder, autosomal recessive periventricular heterotopia with microcephaly (*54*).

As is the case for GBF1, little is known about the regulation of the subcellular localization of BIG1 and BIG2. The association of BIG1 with Golgi membranes was shown to require its N-terminal 560–amino acid region (*47*). Moss, Vaughan and colleagues have reported that BIG2 interacts with regulatory subunits of protein kinase A (PKA) through PKA-anchoring domains within its N-terminal region, and that incubation of cells with a cAMP analogue, 8-Br-cAMP, or an activator of adenylyl cyclase, forskolin, stimulates movement of BIG2 and BIG1 from cytosolic to membrane fractions (*55*). In line with these data, PKA activation was previously reported to enhance association of Arf with Golgi membranes (*56*). Furthermore, the endosome-to-Golgi transport of ricin, a protein toxin, requires PKA activity (*57*), and retrieval of the KDEL receptor from the Golgi to the endoplasmic reticulum requires its PKA-dependent phosphorylation (*58*). Thus, PKA appears to regulate various transport processes from and to the Golgi. It is tempting to speculate that BIG2 might serve to integrate the cAMP-dependent signaling pathway and Arf-dependent membrane traffic.

Structure and catalytic mechanism of the Sec7 domain and its sensitivity to BFA

As described above, BIG1 and BIG2 were originally purified as BFA-sensitive Arf-GEFs (*19*, *52*), whereas GBF1 was identified as a factor that made mammalian

cells BFA-resistant (*33*). All but one (GBF1) of the large Arf-GEFs examined so far have been reported to be sensitive to BFA, whereas all the CYT and EFA6 members examined are insensitive to this drug (reviewed in Ref. *7*).

Early mutagenesis studies have revealed residues of the Sec7 domains critical for the BFA sensitivity (*59*, *60*). Furthermore, kinetic analyses have suggested that BFA acts as an uncompetitive inhibitor that stabilizes an Arf·GDP·Sec7 domain ternary complex, rather than inhibits competitively the interaction of the Sec7 domain with Arf (*47*, *59*). Recent crystallographic studies of the Arf·GDP·Sec7·BFA complex have established the structural basis for the inhibition; namely, BFA binds at the protein-protein interface that involves the switch 1 and switch 2 regions of Arf and the hydrophobic groove of the Sec7 domain and traps the Arf·GDP·Sec7 complex before the nucleotide-release stage (*26*, *61*).

Comparison of the structures of the Arf·GDP, Arf·GDP·Sec7·BFA, Arf·Sec7 (nucleotide-free) and Arf·GTP complexes has suggested a possible model for the nucleotide exchange reaction catalyzed by the Sec7 domain (*26*, *61*). First, the binding of Arf·GDP to the Sec7 domain causes the negative charge of the Glu finger to move toward the nucleotide phosphates and to expel the bound nucleotide and Mg^{2+} ion by steric and electrostatic repulsive effects. The nucleotide-free Arf bound to the Sec7 domain then undergoes a conformational change from a GDP- to a GTP-like state. Finally, the Arf·Sec7 binary complex accepts GTP to dissociate the Sec7 domain.

The structural study has also revealed why Arf-GEF carrying the mutation of the Glu finger to Lys is catalytically inactive and forms a stable complex with Arf·GDP under low Mg^{2+} conditions (*26*, *62*). The Lys residue stabilizes bound GDP by interacting with its α- and β-phosphates, like a Mg^{2+} ion, and thereby interferes with the following conformational change of Arf required for the exchange reaction.

Conclusions and future prospects

Since the first identification of Sec7 domain–containing Arf-GEFs, much progress has been made in understanding their functions in membrane trafficking. As described in this review article, their subcellular localizations have been determined, and the molecular basis for their catalytic mechanism and sensitivity to BFA has been uncovered. However, some important questions remain unanswered.

Because of the presence of six isoforms of Arfs in mammalian cells, it had been previously considered that different Arf isoforms determined where different coat proteins were recruited. However, as the Arf-GEF family has expanded, it has become more likely that Arf-GEFs determine where Arfs are activated, and activated Arfs in turn determine where distinct coated vesicles are formed. Identification of physiological binding partners of Arf-GEFs, proteins or lipids or both, will be required to understand the mechanisms by which they are localized to and activated at specific membrane compartments.

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