Cell. Mol. Life Sci. 65 (2008) 3433 – 3445
1420-682X/08/213433-13
DOI 10.1007/s00018-008-8227-7

Cellular and Molecular Life Sciences

Review

Large ARF guanine nucleotide exchange factors in membrane trafficking

N. Anders and G. Jürgens*

Center of Molecular Biology of Plants, University of Tübingen, 72076 Tübingen (Germany),
Fax: +49 7071 295797, e-mail: gerd.juergens@zmbp.uni-tuebingen.de

Received 25 April 2008; received after revision 26 May 2008; accepted 12 June 2008
Online First 7 July 2008

Abstract. In eukaryotic cells membrane compartments are connected through cargo-selective vesicle trafficking mediating the exchange of components between different organelles. This exchange is essential to maintain their structural integrity and specific composition. A fundamental regulatory step in vesicle formation is the activation of small ARF GTPases by exchanging their bound GDP for GTP, which is a prerequisite for ARF-mediated effector recruitment. Activation of ARFs is catalyzed by the characteristic SEC7 domain of guanine nucleotide exchange factors (ARF-GEFs), which are classified according to their additional protein domains. The only group of ARF-GEFs conserved in mammals, yeast and plants are the large ARF-GEFs. This review summarizes recent findings on the function of large ARF-GEFs, and the use of the inhibitor Brefeldin A as a potent tool in understanding membrane trafficking. Furthermore we highlight common themes and apparent differences in large ARF-GEF function between eukaryotic kingdoms.

Keywords. Membrane traffic, large ARF guanine nucleotide exchange factors (ARF-GEFs), Brefeldin A (BFA), ARF GTPases, ARF-GEF interactors.

Introduction

Small GTPases are molecular switches that utilize GTP binding and hydrolysis as a common mechanism to regulate diverse cellular processes. The activation of GTPases is initiated by guanine nucleotide exchange factors (GEFs), which cause bound GDP to dissociate from the GTPase, leading to its association with GTP [1]. Activated GTPases are capable of recruiting effector proteins, and their activity is terminated by GTP hydrolysis promoted by GTPase-activating proteins (GAPs). The subfamily of ADP-ribosylation factor (ARF) GTPases was first identified as a cellular target of cholera toxin facilitating ADP-ribosylation of the stimulatory Gs alpha subunit of heterotrimeric G proteins. ARFs are primarily involved in regulation of membrane traffic and organization of the cytoskeleton [2, 3]. In contrast to other small GTPases that harbor lipid modifications at the C-terminus, ARFs are myristoylated at their N-terminus to promote membrane association [1]. Interestingly, activation of ARF GTPases is tightly coupled to their membrane association. Similarly, ARF guanine nucleotide exchange factors (ARF-GEFs) are recruited to their target membranes to control ARF activation in space and time. Thus, understanding the functional regulation of ARF exchange factors is crucial to understanding the processes regulated by ARF GTPases.

* Corresponding author.

Table 1. ARF-GEFs in *Saccaromyces cerevisiae* (*S.c.*), *Homo sapiens* (*H.s.*) and *Arabidopsis thaliana* (*A.t.*). ARF-GEFs predicted or shown to be BFA-sensitive are written in boldface, large ARF-GEFs are highlighted in grey. Only one GenBank entry has been cited for each ARF-GEF.

ARF-GEF (S.c.)	Accession number	Size (amino acids)
Gea1p	**NP_012565**	**1408**
Gea2p	**NP_010892**	**1459**
Sec7p	**P11075**	**2009**
Syt1p	**Q06836**	**1226**
Yel1p (Syt2p, ArfGEF5)	P34225	687

ARF-GEF (*H.s.*)	Accession number	Size (amino acids)
BIG1 (p200, ARF-GEF1)	**NP_006412**	**1849**
BIG2 (ARFGEF2)	**NP_006411**	**1785**
BIG3	AAL04174	1770
BRAG1 (IQSEC2, ARFGEP100)	NP_055890	949
BRAG2 (GEP100, IQSEC1)	NP_055684	963
BRAG3 (synArfGEF, IQSEC3)	EAW88981	865
CYH1 (PSCD1)	NP_004753	398
CYH2 (ARNO, PSCD2)	NP_059431	400
CYH3 (GRP1, PSCD3)	NP_004218	399
CYH4 (PSCD4)	NP_037517	394
EFA6A (PSD)	NP_002770	1024
EFA6B (PSD4)	CAD30842	1056
EFA6C (PSD2)	NP_115665	771
EFA6D (PSD3)	NP_056125	1047
FBX8 (FBXO8)	NP_036312	319
GBF1	**NP_004184**	**1859**

ARF-GEF (*A.t.*)	Accession number	Size (amino acids)
BIG1	**At4 g38200**	**1698**
BIG2	**At3 g60860**	**1793**
BIG3 (BIG2, EDA10)	At1 g01960	1750
BIG4	**At4 g35380**	**1706**
BIG5 (AtMIN7)	**At3 g43300**	**1756**
GNL1	At5 g39500	1443
GNL2	**At5 g19610**	**1375**
GNOM (EMB30)	**At1 g13980**	**1451**

ARF-GEFs are characterized by their highly conserved catalytic SEC7 domain that facilitates ARF binding and GDP/GTP exchange, which is named after the first identified yeast ARF-GEF Sec7p. Depending on the presence and homology of additional protein domains, the guanine nucleotide exchange factors are grouped on the basis of their size into small (~40–80kD), medium-sized (~100–150kD) and large (~170–200kD) ARF-GEFs [4, 5]. Genes encoding Sec7 domain-containing proteins are found in all sequenced eukaryotic organisms. Interestingly, only the large ARF-GEFs are conserved between eukaryotic kingdoms, suggesting their involvement in fundamental cellular processes (Fig. 1, Table 1). Apart from the three large ARF-GEFs Sec7p and Gea1/2p, the genome of *Saccharomyces cerevisiae* (hereinafter referred to as yeast) encodes two medium-sized ARF-GEFs Syt1p and Yel1p (Syt2p; Fig. 1A) [4, 6]. Similarly, the human genome encodes three large ARF-GEFs GBF1 and BIG1/2 (Fig. 1B). Interesting-

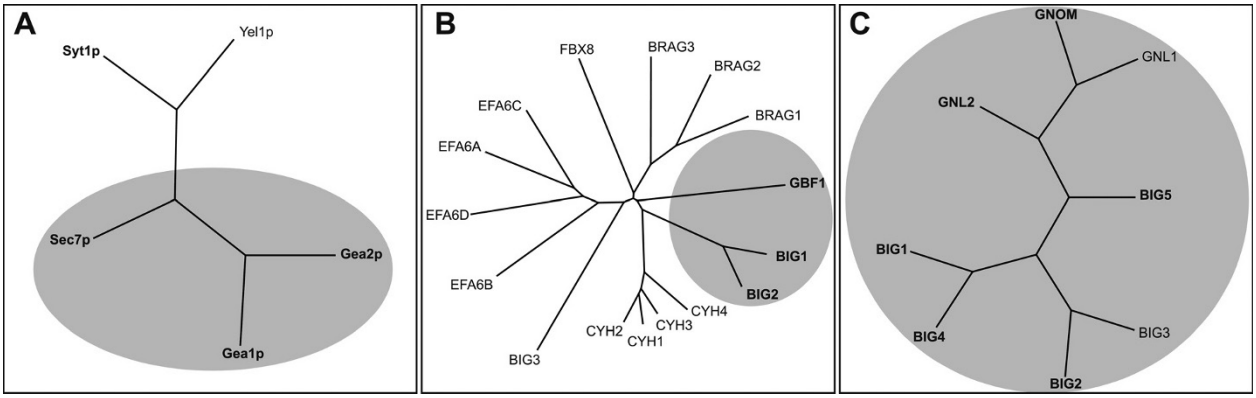

Figure 1. Phylogenetic tree of ARF-GEFs in eukaryotic kingdoms. (*A*) ARF-GEFs in *Saccaromyces cerevisiae.* (*B*) ARF-GEFs in *Homo sapiens.* (*C*) ARF-GEFs in *Arabidopsis thaliana.* The full-length protein sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw) and the phylogenetic tree drawn with TreeView. ARF-GEFs predicted or shown to be BFA-sensitive are in boldface. Note the conservation of large ARF-GEFs highlighted in grey.

ly, another large ARF-GEF BIG3 has been identified, though the sequence is only distantly related to GBF1, BIG1/2 and, moreover, its function is completely unknown [7]. In addition, the human genome encodes a large number of small and medium-sized ARF-GEFs grouped into Cytohesins (CYHs), BFA-Resistant ARF-GEFs (BRAGs), Exchange Factors for ARF6 (EFA6 s) and the single F-Box 8 (FBX8; Fig. 1B) recently reviewed in [7]. Surprisingly, plants do not encode any small or medium-sized ARF-GEFs. Rather, the number of large ARF-GEFs encoded in the genome of *Arabidopsis thaliana (*hereinafter referred to as Arabidopsis) is expanded to eight: GNOM, GNL1/2 and BIG1–5 (Fig. 1C). Simple plant organisms such as algae encode only three large ARF-GEFs, as do yeast and humans [8]. The differences in the ARF-GEF composition between kingdoms and organisms suggests that the encoding genes were multiplied or diversified by neo-functionalization or elaboration of additional ARF-GEF families to meet the increasing trafficking requirements of multicellular organisms [4, 5, 8, 9].

The ARF GDP/GTP guanine nucleotide exchange reaction facilitated by ARF-GEFs can be blocked by the toxic metabolite Brefeldin A (BFA) that is produced by several fungal species in order to associate with host plants [10, 11]. Thus, BFA evolved as a prevalent and potent tool for interfering with membrane traffic. It is mainly the trafficking pathways regulated by large ARF-GEFs that are sensitive to BFA [4]. Genetic, biochemical and structural analysis unraveled the molecular target of BFA in detail [12–16]. These results now allow for manipulation of BFA-sensitivity and resistance of single ARF-GEFs that enables the specific inhibition of vesicle trafficking pathways by molecular engineering. This approach has recently been used to identify the trafficking pathways regulated by a specific ARF-GEF [8, 17].

Here, we review recent progress made in understanding the function and regulation of large ARF-GEFs in eukaryotic kingdoms, as they represent the main target of BFA and, moreover, the only group conserved in eukaryotes.

Effects of the fungal toxin Brefeldin A on membrane trafficking

The fungal toxin Brefeldin A (BFA) exhibits specific effects on membrane trafficking, which established BFA as a potent cell-biological tool. The first effect observed upon addition of BFA to cells is the rapid release of vesicle coat proteins, such as COPI and clathrin coat subunits, into the cytosol [18–20]. In addition, BFA has a strong effect on the integrity of subcellular compartments by inhibiting intracellular trafficking pathways. The most characteristic effect of BFA is the inhibition of secretion and the re-distribution of Golgi-resident membrane proteins to the endoplasmic reticulum (ER) [21–23]. In addition, *trans*-Golgi network (TGN) and endosomal compartments aggregate and fuse with one another; although endosome-to-lysosome transport is impaired, cycling between plasma membrane and endosomes remains functional [24, 25]. Similar effects to those described in human cells were shown in tobacco (*Nicotiana tabacum*) cells [26, 27] and in BFA-permeable mutant yeast strains [28–30]. However, these classical BFA effects were not reproducible in all organisms. Most strikingly, Golgi-resident membrane proteins do not relocate to the ER in Arabidopsis and Mardin-Darby Canine Kidney(MDCK) cells demonstrating BFA-resistance of the Golgi apparatus [8, 9, 17, 31]. These

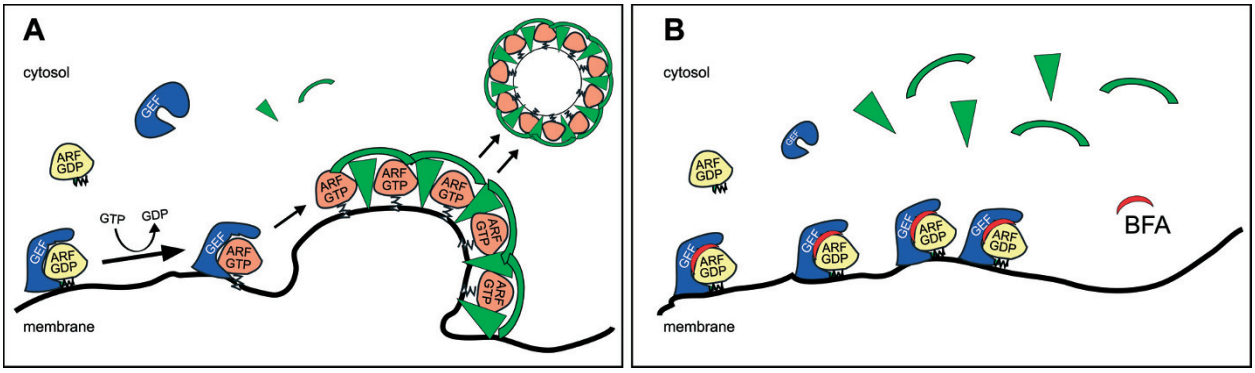

Figure 2. Scheme of ARF GTPase activation by GDP/GTP exchange factors regulating vesicle formation. (*A*) Guanine nucleotide exchange (GDP/GTP) on ARF (yellow/orange) by the guanine nucleotide exchange factor (GEF; blue) leads to the recruitment of vesicle coat components (green) and hence vesicle formation. (*B*) Brefeldin A (BFA, red) traps the ARF●GDP/GEF complex on membranes, preventing GDP/GTP exchange, vesicle coat recruitment and vesicle formation.

differential effects of BFA suggests that BFA has either conserved molecular targets that are differentially sensitive to BFA or that different targets are present in different organisms.

ARF guanine nucleotide exchange and the molecular target of Brefeldin A

Activated ARF GTPases recruit diverse effector proteins to the membrane. In the context of membrane trafficking, the most prominent effectors are vesicle coat components such as coatomer (COPI), clathrin adaptor proteins (APs) and Golgi-localized γ-ear-containing ARF-binding proteins (GGAs) [2]. BFA induces the rapid release of coat components into the cytosol, as it blocks the GDP/GTP exchange on ARFs (Fig. 2). Elegant biochemical and structural analyses unraveled the catalytic exchange mechanism of the SEC7 domain on the ARF protein and the molecular requirements for BFA inhibition [12, 15, 32]. The inactive ARF●GDP is primarily cytosolic. ARF●GDP interacts with the GEF and forms a transient complex at specific target membranes. The conserved so-called glutamate finger within the SEC7 domain of the ARF-GEF is required for the displacement of the bound GDP and its replacement by GTP [33]. Upon GTP loading onto the ARF, an N-terminal myristoylated amphipathic alpha-helix extrudes to mediate ARF membrane association and release of the ARF-GEF into the cytosol [34]. BFA blocks the initial step of the exchange reaction by binding to the interface between the ARF●GDP and the SEC7 domain of the ARF-GEF, trapping the ARF●GDP/SEC7 complex at the membrane [12, 35, 36]. Not all ARF●GDP/SEC7 complexes are accessible to BFA inhibition, as residues of the ARF and the SEC7 domain define a pocket for the binding of BFA and hence BFA-sensitivity or resistance of the complex [37]. Genetic screens uncovered a 40-amino acid region of the SEC7 domain that harbors residues conferring BFA-resistance. Mutating characteristic residues of the GEF leads to altered BFA-resistance/sensitivity of the ARF●GDP/SEC7 complex, which can be used to specifically switch off ARF-GEF function by BFA treatment [8, 13, 16, 17]. Sequence analysis of critical amino acids in the SEC7 domain in conjunction with experimental analysis revealed that all small and medium-sized ARF-GEFs in yeast and humans are resistant to BFA with the single exception of Syt1p [4]. In contrast, all large ARF-GEFs in yeast and humans are BFA-sensitive. In Arabidopsis, however, which only has large ARF-GEFs, there is a presence of those that are BFA-resistant and BFA-sensitive (Table 1, Table 2 and Figure 1C). Studying the subcellular localization and function of large ARF-GEFs will thus lead to a better understanding of the cellular effects of BFA.

Large ARF-GEFs are represented by two subfamilies, the GBF1/Gea/GNOM (GGG) group represented by human GBF1, yeast Gea1/2 and Arabidopsis GNOM and GNOM-like 1/2; and the BIG group, including human BIG1/2, yeast Sec7p and Arabidopsis BIG1–5 (Table 2). The following section summarizes the current knowledge on the cellular function of large ARF-GEFs in yeast, humans and Arabidopsis.

Large ARF-GEFs in the yeast *Saccharomyces cerevisiae*

The first identified large ARF-GEFs that were characterized in yeast are all required for the integrity of membrane compartments [38,39]. The *SEC7* gene was identified in a screen for secretory mutants, shown to be essential for vegetative growth [40]. Sec7p

Table 2. Subcellular localization or presumed site of action of large ARF-GEFs and their response to BFA. To distinguish the BIG from the GBF1/Gea/GNOM (GGG) group the latter is highlighted in grey. *S.c.: Saccaromyces cerevisiae*; *H.s.: Homo sapiens*; *A.t.: Arabidopsis thaliana*; n.d.: not determined; VTC: Vesicular-tubular compartment; TGN: *trans*-Golgi network; PM: plasma membrane; *: experimentally confirmed. Note the presence of BFA-resistant large ARF-GEFs in Arabidopsis.

Large ARF-GEFs (*S.c.*)	Predominant subcellular localization	Predicted response to BFA
Gea1p	VTC, Golgi	Sensitive*
Gea2p	VTC, Golgi	Sensitive*
Sec7p	*trans*-Golgi, TGN	Sensitive*
Large ARF-GEFs (*H.s.*)	**Predominant subcellular localization**	**Predicted response to BFA**
GBF1	VTC, *cis*-Golgi	Sensitive*
BIG1	TGN, (Nucleus)	Sensitive*
BIG2	TGN, recycling endosomes	Sensitive*
Large ARF-GEFs (*A.t.*)	**Predominant subcellular localization**	**Predicted response to BFA**
GNOM	Endosomes, (Golgi)	Sensitive*
GNL1	Golgi, (PM)	Resistant*
GNL2	n.d.	Sensitive
BIG1	n.d.	Sensitive
BIG2	n.d.	Sensitive
BIG3	n.d.	Resistant*
BIG4	n.d.	Sensitive
BIG5	n.d.	Sensitive

localizes to the *trans*-Golgi apparatus and is involved in COPI-mediated intra-Golgi transport [38,39,41–43]. In addition, Sec7p recruits a novel coat complex named exomer to the TGN membrane to facilitate transport to the plasma membrane [44]. The two other large ARF-GEFs of yeast Gea1p and Gea2p were identified as multi-copy suppressors of dominant-negative ARF2 [45]. These two genes are functionally redundant, as neither of them is required for viability, but the double mutant is lethal [39, 45]. Further analysis of temperature sensitive mutants revealed their involvement in ER-Golgi and intra-Golgi transport [46]. However, the two proteins are not completely redundant, as they show differences in localization as well as in genetic interaction with ARF1 [39].

The genome of yeast encodes three ARF GTPases (ARF1–3). Interestingly, all three large ARF-GEFs mediate exchange on ARF1 and ARF2 to activate COPI-dependent vesicle trafficking in the early secretory pathway [38, 39, 45, 47, 48]. Gea1/2p can also activate ARF3, although not required in the context of vesicle formation [49].

Large ARF-GEFs in humans

There are only three large ARF-GEFs in humans. However, alternatively spliced isoforms have been reported, although their functional significance is unknown [5, 50]. Similar to the yeast large ARF-GEFs Gea1/2p, the mammalian ortholog GBF1 localizes to the ER-Golgi intermediate compartment (ERGIC), or the vesicular-tubular compartment (VTC) and the *cis*-Golgi, regulating COPI-dependent vesicle trafficking [51–55]. Depletion of GBF1 by siRNA induces unfolded protein response and cell death, underlining the crucial function of this protein in the early secretory pathway [56]. Surprisingly, however, recent data show that GBF1 recruits GGA to membrane, suggesting an involvement of GBF1 in clathrin-mediated vesicle trafficking [57].

BIG1 localizes to the *trans*-Golgi and TGN, partially overlapping with BIG2 at the TGN, whereas BIG2 additionally localizes to recycling endosomes [52]. Both BIGs promote clathrin-mediated vesicle trafficking by recruiting adaptor proteins, although BIG2 also recruits GGA to membranes [51, 52, 58]. The two distinct BIGs exhibit redundant functions at the TGN as shown by siRNA-mediated knockdown and overexpression of wildtype or dominant-negative versions

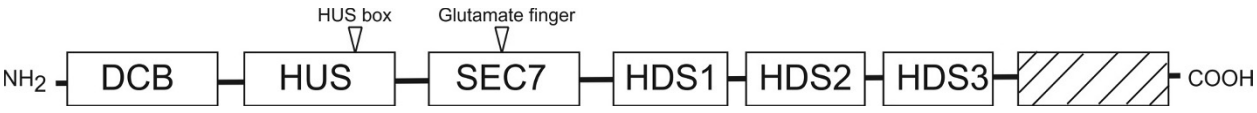

Figure 3. Scheme of domain organization common to large ARF-GEFs. DCB: Dimerisation and Cyclophilin Binding domain; HUS: Homology Upstream of Sec7 domain; HDS1–3: Homology Downstream of Sec7 domains 1–3; according to [5]. The striped box marks the C-terminus that is not as highly conserved. Arrowheads indicate positions of the Hus box motif and the catalytic glutamate finger.

[51, 58–60]. In addition, each BIG protein has its own separate function. BIG1 is required for Golgi integrity and correct glycosylation of integrin β1 [61]. In contrast, BIG2 is involved in recycling of the tumor necrosis factor receptor (TnfR) and the transferrin receptor (TfnR) to the plasma membrane [62, 63]. In addition, the interaction of BIG2 with the γ-aminobutyric acid type-A (GABA) receptor suggests its involvement in secretion [64], which is consistent with the direct interaction of BIG2 with the Exo70 subunit of the exocyst complex [65]. A human hereditary disorder with malformation of the cerebral cortex (autosomal recessive periventricular heterotopia with microcephaly, ARPHM) is associated with mutation(s) in BIG2, demonstrating the requirement of large ARF-GEFs in neuronal cell proliferation and migration of cells during development [66].

The substrate specificity of large ARF-GEFs in humans is not completely clear, as *in vitro*-and *in vivo*-analyses have generated controversial results. GBF1 catalyzes the *in vitro*-exchange on ARF5 [67] whereas ARF1 and ARF4 appear to be the *in vivo*-substrates [68, 69]. Similarly, BIG1/2 catalyze the *in vitro*-exchange on ARF1, ARF3, ARF5 and ARF6 [70–72], whereas, *in vivo* BIG1/2 appear specific for ARF1 and ARF3 in a non-redundant and additive fashion [60, 62, 73].

Large ARF-GEFs in *Arabidopsis thaliana*

The first GBF1/Gea1/2p homolog in Arabidopsis called GNOM was identified in a mutagenesis screen. The *gnom* mutant displays severe defects in early development resulting in lethality, although this ARF-GEF is non-essential for cell viability [74, 75]. Surprisingly, GNOM localizes to endosomal compartments, mediating the polar recycling of the auxin efflux carrier PIN1 to the plasma membrane [17, 76]. GNOM is required for polar auxin transport, which is essential for key developmental processes [17, 77, 78]. Its closest homolog GNL1 mainly localizes to the Golgi apparatus and is required for ER-Golgi transport, although the *gnl1* knock-out leads to minor defects in development [8, 9]. GNOM is capable of functionally replacing GNL1 at the Golgi stack and the double knock-out results in lethality, demonstrating that both ARF-GEFs act redundantly in the early secretory pathway [8]. In addition, GNL1 functions at an early step of endocytosis of specific proteins [9]. Remarkably, Arabidopsis GNL1 is one of the few BFA-resistant large ARF-GEFs and thus accounts for the differences in BFA sensitivity of the Golgi apparatus in Arabidopsis compared to other organisms, such as tobacco, for which the predicted BFA-sensitive NtGNL1 has just been reported [79]. The subcellular localization of all other ARF-GEFs in Arabidopsis is still unknown. However, limited data are available on additional large ARF-GEFs. GNL2 seems to perform a pollen-specific function, as its expression is restricted to the male gametophyte [8]. BIG5 (AtMIN7) is suggested to function in pathogen defense, presumably acting in the late secretory pathway [80], and BIG3 (EDA10) appears to be involved in the development of the female gametophyte [81]. The ARF substrates of Arabidopsis ARF-GEFs are unknown, with the only exception of *in vitro*-GDP/GTP exchange activity of the SEC7 domain of BIG3, formerly also called BIG2, towards ARF1 [82].

Molecular Organization of large ARF-GEFs and their interactors

ARF-GEFs are characterized by their central Sec7 domains that facilitate GDP/GTP exchange on ARFs. Apart from the catalytic Sec7 domain, large ARF-GEFs harbor additional, highly conserved protein domains that are functionally ill-defined [4, 5]: the N-terminal Dimerisation and Cyclophilin Binding (DCB) domain, the Homology Upstream of Sec7 (HUS) domain and three Homology Downstream of Sec7 (HDS1–3) domains (Fig. 3). It is likely that these domains are involved in determining the subcellular localization and membrane association of large ARF-GEFs. In addition, the non-catalytic domains are supposed to be required for the integration of signals in order to regulate ARF activation and hence vesicle trafficking pathways. Diverse interactors of large ARF-GEFs have been identified to date and often the interaction surfaces have been mapped to single domains (Table 3). However, it is still unclear which of

Table 3. Large ARF-GEFs and their interactors. *S.c.: Saccaromyces cerevisiae*; *H.s.: Homo sapiens; R.n.: Rattus norvegicus* and *A.t.: Arabidopsis thaliana*; n.d.: not determined. Interacting protein regions of the GEFs were approximated to established domains [5]. ARF GTPases and viral or bacterial interactors are not listed.

Interactor	Species	Large ARF-GEF	Interaction domain	Reference
AMY-1	*H.s.*	BIG1/2	DCB	[107]
AtCyp19–4	*A.t.*	GNOM	DCB	[83]
Drs2p	*S.c.*	Gea2p	Sec7	[99]
Exo70	*H.s.*	BIG2	DCB-HUS	[65]
FKBP13	*H.s.*	BIG1/2	DCB	[94]
GABA receptor	*R.n.*	BIG2	C-terminus	[64]
GGA	*H.s.*	GBF1	DCB-HUS	[57]
Gmh1p	*S.c.*	Gea1/2p	HDS1–3	[98]
MyosinIXb	*H.s.*	BIG1	HDS3 and C-terminus	[111]
Nucleolin	*H.s.*	BIG1	n.d.	[109]
Nucleoporin 62p	*H.s.*	BIG1	n.d.	[109]
p115	*H.s.*	GBF1	C-terminus	[88]
p90	*S.c.*	Sec7p	n.d.	[117]
PKA	*H.s.*	BIG2	DCB-HUS	[92]
PP1γ	*H.s.*	BIG1/2	n.d.	[104]
Rab1b	*H.s.*	GBF1	DCB	[100]
Sec21p	*S.c.*	Sec7p	HUS-Sec7-HDS1	[38]
Sec24p	*S.c.*	Sec7p	HDS1–3 and C-terminus	[38]
TNFR1	*H.s.*	BIG2	n.d.	[62]

the non-catalytic domains of large ARF-GEFs accomplishes what function and whether these functions are a general feature of large ARF-GEFs or whether they are characteristic for specific vesicle trafficking pathways.

Apart from the SEC7 domain, the best-characterized domain to date is the DCB domain that confers N-terminal dimerisation, initially identified in GNOM by yeast two-hybrid interaction assay [83]. This function is highly conserved and promotes the formation of high molecular-weight protein complexes [45, 84–87]. This is consistent with several interactors being described or suggested to form dimers as well [88–92]. Interestingly, dimerisation of the viral 3A protein is a prerequisite for its binding to GBF1 and its interference with vesicle trafficking [93]. Deletion of the DCB domain or the presence of specific mutations in the DCB domain impairs ARF-GEF function, demonstrating its functional requirement [66, 87]. However, dimerisation seems not to be the key function of the DCB domain [85]. Two immunophilin family members, which act as peptidyl-prolyl *cis/trans* isomerases, were identified as interactors of the DCB domain of human and Arabidopsis large ARF-GEFs [83, 94]. However, both immunophilins harboring an ER-translocation signal peptide were also reported to localize to the lumen of the secretory pathway, therefore the functional relevance of this interaction is unclear [85, 95].

Mutations in the highly conserved motif (HUS box) in the HUS domain of Gea2p severely affect viability, demonstrating the functional requirement of the HUS domain [5, 47]. In contrast, deletion of the major part of the three HDS domains has comparatively minor effects on the functionality of GNOM [77].

Membrane association of large ARF-GEFs and functional regulation

Large ARF-GEFs reversibly associate with target membranes, continuously cycling on and off [68, 96, 97]. Surprisingly, they do not harbor a characterized membrane-association domain, which contrasts greatly with small- and medium-sized ARF-GEFs that possess a pleckstrin-homology (PH) domain mediating membrane association through interaction with specific lipids and ARF proteins [7]. Several integral membrane proteins have been identified as interactors of large ARF-GEFs (Table 3). Gea1p and Gea2p interact with the Golgi membrane protein Gmh1p. However, deletion of Gmh1p did not drastically affect

membrane association of Gea1/2p [98]. Similarly, GBF1 interacts with the tether protein 115p and Gea2p interacts the phosphatidylserine flippase Drs2p, but although both are integral membrane proteins, they do not mediate membrane association of large ARF-GEFs [88, 99]. Monetta et al. 2007 identified Rab1b as an interactor of the DCB domain, and Rab1b depletion by siRNA or expression of mutant Rab1b demonstrate its requirement for membrane recruitment of GBF1 and COPI [100, 101]. This is consistent with the genetic interaction of the yeast Ypt/rabs with Sec7p and Gea1/2p [102] and the idea of a requirement of Ypt/rabs also in the budding process of vesicles [103]. Thus, diverse Rab proteins that mark different membranes of the endomembrane system might function as receptors for large ARF-GEF membrane recruitment.

In addition, the DCB domain has been described as interacting with the adjacent HUS domain of human and Arabidopsis large ARF-GEFs, although in Arabidopsis the N-terminal part of the SEC7 domain is involved in this interaction as well [85, 87]. This interaction is essential for the function of the ARF-GEF GNOM and, moreover, is required for ARF-GEF membrane association, as mutations abolishing this interaction affect membrane association of large ARF-GEFs [47, 85]. This finding is consistent with data demonstrating that the DCB domain is required but not sufficient for membrane association of large ARF-GEFs [100]. Interaction of the DCB domain with the HUS (and the Sec7) domain might occur intra- or intermolecularly, hence large ARF-GEFs might not only form dimers via their DCB domains but tetrameric complexes by an additional intermolecular interaction [85, 87]. However, it is unknown whether the interaction of the DCB domain with the HUS (and the SEC7) domain is regulated in order to control ARF-GEF membrane association and hence ARF activation.

Not much is known about the regulatory control of large ARF-GEF function at all. Human BIG1/2 bind to protein kinase A (PKA) via conserved PKA-binding sites and interact with protein phosphatase 1 γ (PP1γ) [92, 104]. PKA-catalyzed phosphorylation of BIG2 decreases the exchange activity, which is restored by PP1γ-catalyzed dephosphorylation. In addition, the less active phosphorylated BIG1/2 seems more abundant in the cytosol, whereas the less phosphorylated, more active proteins accumulate in membrane fractions, suggesting a regulation of membrane association by phospohorylation and dephosporylation [104]. Sec7p has been reported to be a phosphoprotein as well [105]. However, there are no known conserved phosphorylation sites of large ARF-GEFs that are involved in the regulation of ARF-GEF activity and membrane association. Surprisingly, ARF1 membrane recruitment is induced by PKA activity, presumably mediated by phosphorylated target proteins in the membrane [106]. In addition, human BIG2 interacts with AMY-1 (associate of Myc-1) and recruits it to *trans*-Golgi membranes. AMY-1 appears to interact with A-kinase-anchoring proteins (AKAPs), suggesting that AMY1 might modulate the function of PKA or serve as a scaffold for other proteins [107].

Phosphorylation has been reported to play a role in functional regulation of large ARF-GEFs under specific conditions. Upon glucose depletion, AMP-activated protein kinase regulates GBF1 activity by phosphorylation [108]. Phosphorylation of BIG1 by PKA under serum-starvation conditions regulates translocation of BIG1 to the nucleus where it precipitates with nuclear proteins [109, 110]. However, the function of ARF-GEFs in the nucleus is entirely unknown.

Large ARF-GEFs – more than ARF activators?

The classical view of ARF-GEF function is the activation of ARF GTPases. ARF GTPases, in turn, are crucial for initiating downstream events required for vesicle formation. In addition, ARF GTPases have an important function in the regulation of actin cytoskeleton assembly [3]. Interestingly, activation of ARF3 by Gea1/2p is required for the organization of the actin cytoskeleton, which might involve the activation of Rho GTPases [49]. However, recent data suggest that large ARF-GEFs are not required only for the activation of ARF GTPases initiating ARF-mediated signaling cascades. Human BIG1 interacts with the Rho-GTPase-activating protein (Rho-GAP) Myosin IXb. The interaction of Myosin IXb with BIG1 inhibits its GAP activity, probably due to the competition of BIG1 with the Rho GTPase for the Rho-GAP binding site, suggesting that Rho activation is regulated by the local concentration of BIG1 [111]. Moreover, large ARF-GEFs interact directly with vesicle coat components, suggesting a function in vesicle coat selection. Different protein domains seem to mediate these interactions. Co-immunoprecipitation experiments demonstrated an interaction of GBF1 with GGA that was mapped to the DCB and HUS domains by yeast two-hybrid analysis [57]. In addition, *in vitro*-binding experiments showed that a region of Sec7p approximately corresponding to the HUS-Sec7-HDS1 domains interacts with COPI and, more surprisingly, a region within Sec7p approximately corresponding to HDS1–3 and the C-terminus interacts with COPII coat components [38]. In

addition, large ARF-GEFs might also be involved in the selection of cargo, as the GABA receptor directly interacts with the C-terminal domain of BIG2 in rat [64]. However, direct interaction of other cargo proteins with large ARF-GEFs and specificity in this interaction remains to be investigated.

ARF-GEFs as targets and tools during pathogen infection

Diverse pathogens exploit host membrane trafficking to create a favorable environment for survival and replication. The enteroviruses, associated with several human and mammalian diseases, produce a 3A protein that inhibits COPI-mediated (ER)-to-Golgi trafficking, most likely to suppress antiviral host cell responses [112, 113]. Recent work showed that the membrane protein 3A interacts with the DCB and the HUS domain of GBF1, traps it on membranes and hence inhibits ARF1 activation [87, 93]. Likewise, a closely related enterovirus 3A protein and the 3CD protein are able to recruit GBF1 and BIG1/2 to membranes, respectively [114]. However, the combinatorial expression of 3A and 3CD leads to an enhancement of ARF1 activation during infection, promoting the formation of viral replication complexes [114]. There is no molecular explanation for the conflicting observations. Nonetheless, the fact that ARF-GEFs are targeted by viral protein underlines their central role in the maintenance of endomembrane organization.

Bacterial pathogens have evolved different ways of interfering with large ARF-GEF function and hence membrane trafficking of the host cell. The HopM1 virulence protein of the plant pathogen *Pseudomonas syringae* recruits BIG5 (AtMIN7) in Arabidopsis and promotes its ubiquitination and subsequent degradation by the host proteasome to inhibit host vesicle trafficking pathways [80]. In addition, the pathogens *Leginella pneumophila* and *Rickettsie prowazekii* express a SEC7-containing virulence protein RalF [115], which is used to subvert mammalian host vesicle trafficking and to create a stable vacuole in which to replicate. Interestingly, the RalF ARF-GEF harbors a novel C-terminal domain, called SEC7 capping domain (SCD) that interacts with the SEC7 domain. The interaction of the SCD domain with the SEC7 domain sterically hinders ARF binding and enhances RalF catalytic activity, providing the first evidence that domain reorganization is a required step in the activation of ARF-GEFs [116].

Conclusions and future perspectives

Large ARF-GEFs are highly conserved key players of vesicle trafficking pathways in all eukaryotic kingdoms. The localization and cellular function of Golgi-localized large ARF-GEFs has been extensively studied. In contrast, detailed analyses of the diversified large ARF-GEF family members in Arabidopsis trafficking are still missing.

Major questions regarding both general and specific functions of large ARF-GEFs remain unanswered. Molecular and functional characterization of the non-catalytic protein domains and their interactors is required to unravel upstream and downstream effects and moreover determine specificity cues in vesicle trafficking. Future research will have to elucidate what factors regulate large ARF-GEF function, how incoming signals are integrated and transduced, and how large ARF-GEFs are specifically recruited to, and associated with, membranes to activate ARF substrates. In addition, large ARF-GEFs might also function in other cellular processes besides vesicle trafficking, as ARF-GTPases are regulators of cytoskeleton dynamics, although this field is largely unexplored. Analyzing additional roles of large ARF-GEFs might provide important insight into the molecular regulation and coordination of these fundamental cellular processes.

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