Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors

Catherine L. Jackson and James E. Casanova

ARF proteins are important regulators of membrane dynamics and protein transport within the eukaryotic cell. The Sec7 domain is ~200 amino acids in size and stimulates guanine-nucleotide exchange on members of the ARF class of small GTPases. The members of one subclass of Sec7-domain proteins are direct targets of the secretion-inhibiting drug brefeldin A, which blocks the exchange reaction by trapping a reaction intermediate in an inactive, abortive complex. A separate subclass of Sec7-domain proteins is involved in signal transduction and possess a domain that mediates membrane binding in response to extracellular signals.

Catherine Jackson is in the Service de Biochimie et Génétique Moléculaire, Bat. 142, CEA/Saclay, F-91191 Gif-sur-Yvette Cedex, France. E-mail: cathy@jonas.saclay.cea.fr
James Casanova is in the Dept of Cell Biology, Box 439, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA. E-mail: jec9e@virginia.edu

The ADP-ribosylation factors (ARFs) are a family of small, ubiquitously expressed Ras-like GTPases that are central to many vesicular transport processes in eukaryotic cells[1]. Three ARFs are expressed in the yeast *Saccharomyces cerevisiae*, whereas six have been identified in mammalian cells[2]. Yeast ARF1 and ARF2 are 96% identical and are functionally interchangeable: deletion of both *ARF1* and *ARF2* is lethal, but the single-deletion strains are viable. Yeast ARF1 and ARF2 are 77% identical to human ARF1, and 69% identical to human ARF5, and function in transport through the endoplasmic-reticulum–Golgi (ER–Golgi) and endosomal systems[3]. Yeast ARF3 is not essential for viability, and probably corresponds to mammalian ARF6[4].

Mammalian ARFs can be subdivided into three classes based on sequence. The class-I ARFs (ARFs 1–3) are currently the best understood and have been shown to regulate the assembly of several types of vesicle coat complexes including COPI on the Golgi apparatus, clathrin–AP1 on the *trans*-Golgi network (TGN) and clathrin–AP3 on endosomes[2]. Little is known about the function of class-II ARFs (ARFs 4, 5). ARF6 (the only class-III ARF) is known to be located on the plasma membrane and a subpopulation of endosomes[5], but its precise function at these sites remains unclear. In some cell types, ARF6 regulates endocytosis and membrane recycling as well as aspects of cytoskeletal actin assembly[6].

Like other GTPases, ARFs cycle between active GTP-bound and inactive GDP-bound conformations. For most ARFs, the GDP-bound form is primarily cytosolic, whereas ARF–GTP is membrane bound (ARF6 was originally thought to associate constitutively with membranes, but this has recently been called into question[7,8]). ARFs are myristoylated at their N-terminus, at the end of a 17-residue amphipathic α helix. Early models of ARF function had proposed that the myristyl group and the helix would be retracted into the core of the protein in the GDP-bound conformation; upon GTP binding, they would be extended outward, allowing insertion of the myristate into the membrane bilayer[9]. However, subsequent studies established that the myristate group is exposed and interacts with phospholipids when ARF is in the GDP-bound conformation. This weak but measurable membrane association is completely abolished if the myristate is removed[10]. The ARF–membrane interaction is stabilized in the GTP-bound form by a conformational change in the N-terminal helix that exposes several hydrophobic residues, including Leu8 and Phe9 (which are buried inside the ARF protein in its GDP-bound form), and allows their insertion into the membrane[11]. This interpretation of the role of the helical domain becomes important in understanding the mechanism of ARF nucleotide exchange (Box 1).

The Sec7 family of proteins

ARFs require accessory proteins, referred to as guanine-nucleotide-exchange factors (GEFs) to catalyse the exchange of GDP for GTP, which otherwise occurs very slowly under physiological conditions. Although the existence of such an activity in Golgi membranes was first demonstrated in 1992[12,13], it was not until 1996 that the first ARF GEFs were identified using a genetic selection in *S. cerevisiae*[14]. Since then, it has become apparent that the ARF GEFs constitute a large and surprisingly diverse family of proteins[4] (Fig. 1). Although highly divergent in their overall sequence, these proteins share one common feature – a region of roughly 200 amino acids with strong homology to the yeast protein Sec7p, which has come to be known as the Sec7 domain.

Chardin and colleagues established that guanine-nucleotide-exchange activity resides within the Sec7 domain and that this domain alone is sufficient for exchange activity[15]. The divergent sequences outside the Sec7 domain might play some role in determining substrate specificity, either directly[16] or indirectly by targeting individual GEFs to specific membrane sites. Given the high degree of phylogenetic conservation of the ARFs, it is not surprising that proteins containing Sec7 domains have been identified in a wide variety of organisms, including yeast, plants, protozoa, worms, flies and mammals. The initial studies demonstrating the existence of a Golgi-membrane-associated ARF GEF activity also showed that this activity was completely inhibited

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BOX 1 – PHOSPHOLIPIDS AS PARTNERS IN THE NUCLEOTIDE-EXCHANGE REACTION

ADP-ribosylation factor (ARF) can interact weakly with membrane phospholipids when bound to GDP, whereas its GTP-bound form is tightly membrane bound owing to the interaction of the amphipathic N-terminal helix with the lipid bilayer. It is likely that, in the exchange reaction, membrane interaction initiates the conformational change that is completed by the guanine-nucleotide-exchange factor (GEF).

Lipids alone can stimulate exchange on ARF1 in the absence of a GEF and hence are capable, on their own, of displacing the N-terminal helix[10]. However, this reaction is extremely slow compared with that catalysed by the addition of a Sec7-domain GEF, which accelerates nucleotide exchange on ARF by four to five orders of magnitude[23]. Despite this powerful catalytic action of Sec7-domain GEFs on ARF, the Sec7 domain of ARNO alone, in the absence of lipids, cannot stimulate exchange on full-length myristoylated ARF1, and it is not possible even to isolate a complex between these two proteins in solution in the absence of lipids[58].

These results strongly suggest that lipids are required first, to extract the N-terminal helix. This then allows the GEF to engage ARF in a productive complex. The tight association of the N-terminal helix with the lipid bilayer (characteristic of the GTP-bound form of ARF) occurs very early in the exchange reaction, before GDP is released from the ARF–GEF complex[58]. These results correlate well with the striking observation that the structure of nucleotide-free ARF1 in the Sec7-domain–ARF1 complex closely resembles the GTP-bound conformation[19]. Although a truncated form of ARF1 lacking the N-terminal helix was used in this study to improve crystallization, the binding pocket for the N-terminus no longer exists in the complex owing to the extrusion of loop β2-λ3-β3, suggesting that the helix would in fact be extended. This novel and elegant mechanism of exchange ensures that the activation of ARF by a Sec7-domain GEF occurs exclusively at the surface of a membrane (where the GEF is localized) and not in the cytoplasm.

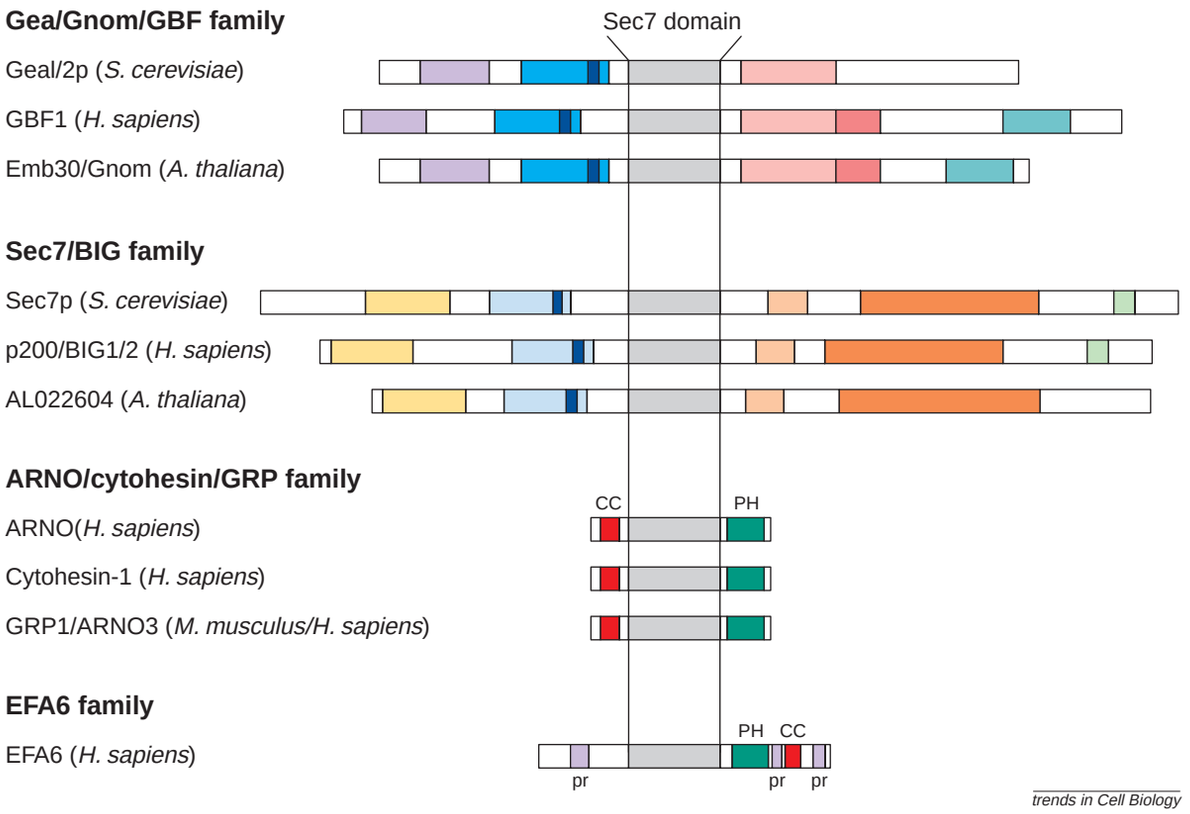

FIGURE 1

The Sec7 family of proteins. Representative members of four different subfamilies of Sec7-domain proteins are shown, with the Sec7 domains represented by grey boxes. For the high-molecular-weight proteins, coloured boxes represent regions showing a significant level of sequence similarity; the functions of these domains have not yet been determined. There is only one motif (I/V/L–N–F/L/Y–D–C) common to all members of both of these subfamilies, and this is represented by a dark-blue box upstream of the Sec7 domain. Sequence comparisons were performed using the PIMA multiple-sequence-alignment program at the BCM Search Launcher (`http://www.hgsc.bcm.tmc.edu/SearchLauncher/`). The coiled-coil (CC) and pleckstrin-homology (PH) domains of the ARNO–cytohesin–GRP and EFA6 subfamilies are indicated. The proline-rich regions of EFA6 are designated 'pr'.

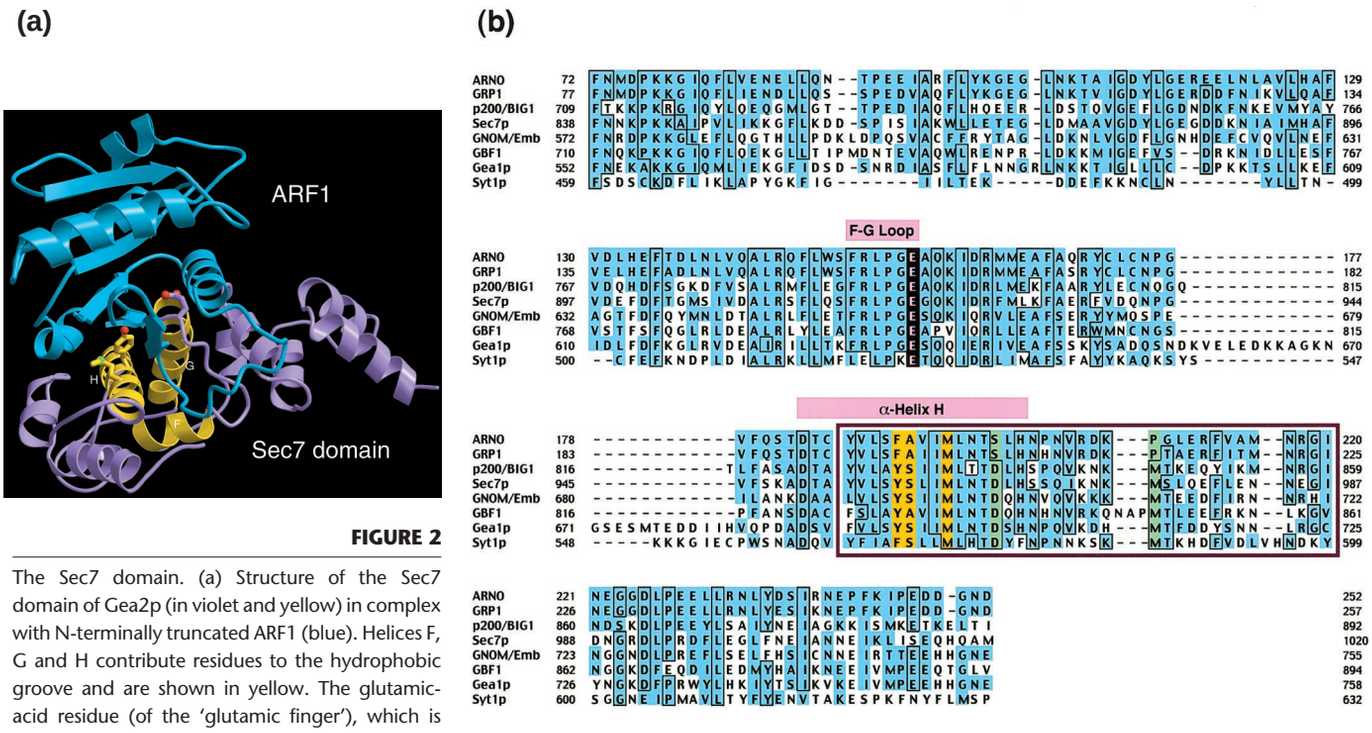

FIGURE 2

The Sec7 domain. (a) Structure of the Sec7 domain of Gea2p (in violet and yellow) in complex with N-terminally truncated ARF1 (blue). Helices F, G and H contribute residues to the hydrophobic groove and are shown in yellow. The glutamic-acid residue (of the 'glutamic finger'), which is essential for guanine-nucleotide-exchange-factor activity, is shown in ball-and-stick representation (purple). The residues of the hydrophobic groove critical for the brefeldin-A (BFA) response both *in vitro* and *in vivo* are also shown in ball-and-stick representation (yellow). (Figure courtesy of Jonathan Goldberg.) (b) Sequence alignment of eight Sec7 domains. Identical residues are boxed and homologous residues are in blue. The essential glutamate residue (corresponding to Glu156 of ARNO) is highlighted in pink. The 35-amino acid region (boxed in purple) is the subdomain identified by mutagenesis studies as crucial for determining sensitivity to BFA. The M699L mutation of Gea1p confers resistance to BFA both *in vivo* and *in vitro*[54]; the corresponding residue is highlighted in yellow in the alignment. The F190Y-A191S two-amino-acid substitution in the ARNO Sec7 domain (highlighted in yellow) dramatically increases the sensitivity to BFA of its exchange activity on ARF1 both *in vitro* and *in vivo* when introduced into the Gea1–ARNO–Gea1p chimera[54]. Similarly, introduction of the S199D,P209M double mutation (the corresponding residues are highlighted in green in the alignment) into cytohesin-1 rendered it BFA sensitive *in vitro*[55]. The alignment was performed using the ClustalW multiple sequence alignment program at the BCM Search Launcher (`http://www.hgsc.bcm.tmc.edu/SearchLauncher/`).

by the fungal toxin brefeldin A (BFA)[12,13]. The molecular basis of this inhibition is now understood and is described in detail below.

Structure of the Sec7 domain

The crystal structure of the isolated ARNO Sec7 domain has been determined at ~2 Å resolution[17,18]. The domain consists of ten α-helices (A–J) arranged in an elongated cylinder (Fig. 2a). A prominent feature of the domain is the presence of a deep hydrophobic groove in the central region (comprising α-helices F, G and H); together with the hydrophilic loop between helices F and G, this forms the binding site for nucleotide-free ARF[19] (Fig. 2a). The sequence of the F–G loop (FRLPGE) is the most highly conserved motif among Sec7 family members (Fig. 2b) and is invariant in all but two of those currently characterized: in Syt1p, the corresponding sequence is LELPKE[20]; in EFA6, it is LALMGE[21].

A second highly conserved motif makes up most of α-helix H and contains a large number of solvent-exposed hydrophobic residues (Fig. 2b). Point mutations within these conserved motifs dramatically reduce exchange activity, demonstrating that this region constitutes the active site of the Sec7 domain[17,18,22]. A more detailed mechanistic analysis by Bruno Antonny and colleagues led them to propose that the invariant glutamate at the C terminus of the F–G loop (E156 in ARNO) forms a 'glutamic finger' that inserts into the nucleotide-binding fold, displacing the coordinating Mg^{2+} ion and possibly the β-phosphate of the bound GDP[23]. Interestingly, a charge-reversal mutation in this glutamate residue identified in a mutant allele of the *Arabidopsis thaliana* protein GNOM/Emb30 results in dramatic developmental defects[24], and a similar mutation introduced into the ARNO[17,18,25], EFA6[21] and cytohesin-1[22] Sec7 domains reduces exchange activity to background levels.

The crystal structure of a complex between the Sec7 domain of Gea2p and nucleotide-free ARF1[19] supports this model, indicating that the glutamate side chain comes within 3 Å of the β-phosphate site and is likely to exert both steric and electrostatic repulsive forces on the bound nucleotide. The Sec7 domain extensively engages both the switch-1 and the switch-2 regions of the ARF molecule, which undergo substantial conformational changes that allow the glutamic finger access to the nucleotide-binding site[19]. The structure of nucleotide-free ARF1 in complex with the Sec7 domain is already very similar to that of the GTP-bound form, which has important implications for understanding the mechanism of exchange (Box 1).

High-molecular-weight GEFs

Members of the Sec7 family characterized to date can be subdivided into two major classes based on

sequence similarities and functional differences. The large (>100 kDa) ARF GEFs have orthologues in all eukaryotes examined and hence are probably involved in evolutionarily conserved aspects of membrane dynamics and protein transport. The smaller (<100 kDa) family members do not have orthologues in *S. cerevisiae*, whose complete genome has been sequenced, suggesting a function specific to higher eukaryotes. The >100 kDa ARF GEFs can be further subdivided into distinct classes (Fig. 1). The first includes yeast Gea1p and Gea2p, *Arabidopsis* GNOM/Emb30p, and human and hamster GBF1; the second class includes yeast Sec7p and mammalian BIG1 and BIG2. A third yeast Sec7-domain protein, Syt1p, shares little sequence similarity with members of either of these classes and so it is possible that it represents a third distinct class[20]. No higher-eukaryotic homologues of Syt1p have yet been identified.

The *S. cerevisiae* Gea1p and Gea2p proteins share 50% identity and are functionally redundant: yeast strains carrying a deletion of either *GEA1* or *GEA2* have no growth or secretion defect so-far identified, whereas the double-deletion strain is nonviable[14]. Similarly, Sec7p function is essential in yeast[26]. The temperature-sensitive mutants *gea1ts*, *gea2Δ* and *sec7ts* have defects in ER–Golgi and intra-Golgi transport[14,27]. Neither Gea1p nor Gea2p can replace Sec7p functionally *in vivo*, and, vice versa, Sec7p cannot compensate for the loss of Gea1p and Gea2p, indicating that each must have a distinct role within the cell. The *Arabidopsis* GNOM/Emb30p protein can restore growth to a *gea1ts gea2*Δ mutant, suggesting that it is a functional orthologue of the Gea1 and Gea2 proteins[28]. In *Caenorhabditis elegans*, there is only one ORF with extensive sequence similarity to Gea1p, Gea2p, GNOM and GBF1; this is called C24H11.7 (accession number Z81475), but no functional information is yet available for it. Although a number of sequences showing a high level of similarity to Sec7p throughout their lengths have been identified[29] (Fig. 1), only Sec7p, BIG1 and BIG2 have been characterized functionally.

GBF1 was originally identified in an attempt to clone the factor responsible for the BFA resistance of a mutant CHO cell line[30]. It confers BFA resistance when overexpressed in mammalian cells and co-localizes with β-COP to the Golgi apparatus[31]. Golgi membranes purified from cells overexpressing GBF1 have the same level of nucleotide-exchange activity on mammalian class-I ARFs as control cells expressing the endogenous level of GBF1, but, whereas the latter is severely inhibited by BFA, the former is BFA resistant[31]. This interesting observation correlates with the fact that Golgi membranes from GBF1-overexpressing cells recruit the vesicle-coat complex COPI in a BFA-resistant fashion. Moreover, in intact cells overexpressing GBF1, the Golgi apparatus is not affected by concentrations of BFA that completely disassemble the Golgi of control cells. These observations all point to a role for GBF1 in class-I ARF function *in vivo*. Surprisingly, it was found that partially purified His6-tagged GBF1 was unable to catalyse GDP–GTP exchange on class-I ARFs *in vitro* under physiological conditions but could catalyse exchange on ARF5 (a class-II ARF)[31].

GNOM/Emb30 is the protein product of the gene mutated in the *gnom* pattern-formation mutant of the *Arabidopsis* embryo[32]. Mutants in the *gnom* gene have a number of phenotypes, including cell-polarity defects and inappropriate positioning of the cell-division plane, starting as early as the first embryonic division[28,32]. As described above, one such *gnom* mutation, E658K, abolishes ARF nucleotide-exchange activity[24]. These results demonstrate a role (either direct or indirect) for an ARF GEF in the establishment and maintenance of cell polarity.

Sec7p was first identified by Novick and Schekman in a selection for *S. cerevisiae* secretion-defective mutants. Sec7p is a Golgi-localized protein in yeast[33] and plays an important role in ER–Golgi and intra-Golgi transport[27]. Sec7p is found in both membrane-bound and soluble forms and is phosphorylated *in vivo*, although the functional significance of this modification is not known[33]. BIG1 (also called p200–GEP) is a Golgi-localized protein in mammalian cells, and this localization is mediated by a region within the N-terminal third of the BIG1 protein[29]. BIG1 and BIG2 catalyse nucleotide exchange most efficiently on class-I ARFs *in vitro* and are also active on ARF5 but do not use ARF6 as a substrate[34,35]. For BIG1, a region ~100 amino acids upstream of the Sec7 domain is required as well as the Sec7 domain itself to obtain the same level of *in vitro* GEF activity as for the full-length protein[29,34].

Low-molecular-weight GEFs

The second subfamily of smaller ARF GEFs contains the proteins ARNO[15], cytohesin-1[36], GRP1/ARNO3[37,38] and EFA6[21]. ARNO, cytohesin-1 and GRP1/ARNO3 are closely related in size (45–50 kDa) and sequence (77% identity), and share a common domain structure (Fig. 1). The *C. elegans* genome also encodes a protein of similar size and domain organization (KO6H7.4). The N-terminal ~60 amino acids form a coiled-coil domain that, in ARNO, mediates homodimerization of the molecule[15]. This is followed by the central Sec7 domain and an immediately adjacent pleckstrin-homology (PH) domain. The PH domain appears to mediate membrane association by binding to either phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P_3] or phosphatidylinositol (4,5) diphosphate [PtdIns(4,5)P_2]. This interaction dramatically stimulates the rate of ARF nucleotide exchange by concentrating the reactants at the membrane surface[39].

Although PtdIns(4,5)P_2 was originally found to be sufficient for both membrane recruitment of ARNO and activation of ARF nucleotide exchange[15], several groups have demonstrated a significant preference by the ARNO[40], GRP1[41] and cytohesin-1[42] PH domains for PtdIns(3,4,5)P_3 (or its soluble head group) over PtdIns(4,5)P_2 *in vitro*. Moreover, transient recruitment of ARNO[40], GRP1[43,44] and cytohesin-1[45] to the plasma membrane in response to phosphoinositide-3-kinase agonists has been demonstrated, and, in each case, recruitment was blocked by the phosphoinositide-3-kinase inhibitors wortmannin and LY294002. These data suggest that members of the ARNO–cytohesin–GRP subfamily of ARF GEFs can be

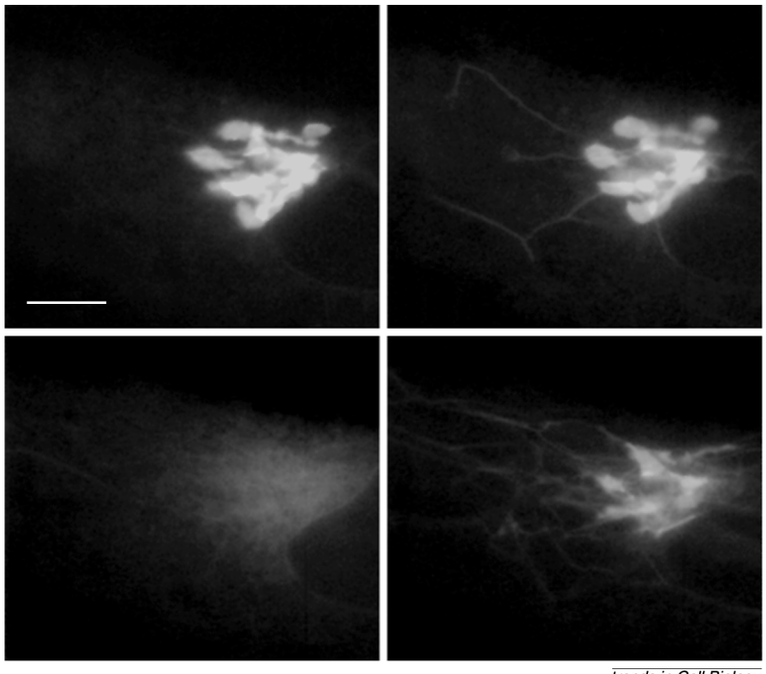

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FIGURE 3

A time course of Golgi disassembly in the presence of brefeldin A (BFA). GFP–galactosyltransferase-expressing HeLa cells were treated with 1 μg ml^{-1} BFA and were visualized by fluorescence microscopy in real time. The sequence runs anticlockwise from the upper left corner, and the images show one cell photographed at 0, 1:36, 4:33 and 4:57 minutes beginning 4 minutes after BFA addition. Bar, 6 μm. (Figure courtesy of Jennifer Lippincott-Schwartz.)

recruited to membrane sites by local production of PtdIns(3,4,5)P_3 *in vivo*.

The C terminus of these proteins includes a fourth domain, variable in length, that contains a large proportion of basic amino acids. In ARNO and cytohesin-1, this polybasic region is interrupted by a consensus protein-kinase-C (PKC) site, which in ARNO is phosphorylated *in vivo*[46]. The polybasic domain cooperates with the PH domain to enhance the binding of cytohesin-1 to PtdIns(3,4,5)P_3, presumably through electrostatic interactions with the negatively charged lipids[45]. The presence of the PKC site in ARNO and cytohesin-1 suggests a mechanism by which the negatively charged phosphate reduces membrane affinity by destabilizing such electrostatic interactions. In support of this hypothesis, binding of recombinant ARNO to phosphoinositide-containing liposomes is dramatically reduced by either PKC phosphorylation or substitution of the phosphorylated serine (S392) with glutamic acid[47]. Interestingly, the lack of a corresponding site in GRP1 implies that it would not be subject to similar regulation. These results and those described above support the idea that the small ARF GEFs are involved in signal-transduction pathways originating at the cell surface. Several lines of evidence suggest that this is accomplished through direct activation of ARF at the plasma membrane as a result of plasma-membrane localization of the small ARF GEFs[25,40,43–45,48], although an additional role for ARNO and ARNO3 at the level of the Golgi has also been suggested[6].

What is the physiological substrate of ARNO, cytohesin-1 and GRP1/ARNO3 at the plasma membrane? ARF6 is an obvious candidate, based on its reported localization to the plasma membrane and a subpopulation of endosomes[6]. In support of this hypothesis, both ARNO[25] and GRP1/ARNO3[44] can catalyse nucleotide exchange on ARF6 *in vitro*, as well as on ARF1. Expression of ARNO in HeLa cells induces changes in the organization of the actin cytoskeleton similar to those caused by the activation of ARF6. ARNO expression alone resulted in a loss of actin stress fibres, but subsequent treatment of ARNO-expressing cells with phorbol esters dramatically stimulated the formation of actin-rich structures resembling lamellipodia[46].

Moreover, Czech and colleagues have shown that both ARF6 and GRP1 are recruited into membrane ruffles following treatment of cells with insulin. By direct analysis of nucleotides bound to immunoprecipitated ARF, they also demonstrated that expression of GRP1 stimulates GTP loading of ARF6 in intact cells[44]. Does this mean that ARF1 is not a substrate for these plasma-membrane GEFs *in vivo*? Certainly not. There are numerous examples of ARF1 recruitment to membranes in response to the ligation of cell-surface receptors[49,50], and recent evidence suggests that ARF1 plays a role in the assembly of focal adhesions[51]. It is therefore possible that both ARF6 and ARF1 are activated by ARNO, cytohesin or GRP1 in response to cell-surface signalling events.

Franco *et al*. described a novel Sec7-family member specific for ARF6 *in vitro*, termed EFA6[21] (Fig. 1). It contains three proline-rich regions that could interact with other proteins. The Sec7 domain of EFA6 is only 30% identical to that of ARNO, compared with the 42% identity between those of ARNO and Sec7p. Strikingly, the conserved motifs that form the catalytic site are less well conserved in EFA6[21], which might explain its distinct substrate specificity. Although EFA6 is expressed primarily in the brain, several other known sequences appear to encode related proteins with similarly divergent Sec7 domains and that might be expressed more ubiquitously[21].

Consistent with its ability to activate ARF6 *in vitro*, expression of EFA6 induced membrane ruffling in CHO cells and also inhibited the uptake of fluorescent transferrin. Unlike ARNO, cytohesin and GRP1, which are transiently recruited to the plasma membrane in response to phosphoinositide production, EFA6 appears to be primarily membrane bound and thus might be constitutively active. Interestingly, homology searches reveal that the EFA6 PH domain is closest in sequence to that of β spectrin, which, unlike ARNO and its relatives, has an equivalent affinity for PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. The relative abundance of PtdIns(4,5)P_2, even in resting cells, might therefore explain the constitutive interaction of EFA6 with membranes.

Determinants of BFA sensitivity lie within the Sec7 domain

Treatment of intact cells with BFA results in loss of organelle identity and in a block to many transport steps[52]. The most spectacular effect is the rapid

disassembly of the Golgi complex and the redistribution of resident Golgi markers into the ER, which takes place within minutes of BFA treatment (Fig. 3). Similarly, the TGN fuses with the early (sorting) endosome[52]. One of the earliest effects of BFA treatment is the dissociation of a large number of peripherally associated membrane proteins into the cytoplasm, including COPI from the Golgi membranes[12,13]. Consistent with the idea that the Sec7 family of exchange factors are direct targets of BFA, a number of these proteins (including Gea1p and Gea2p[14], GNOM/Emb30[28], Sec7p[53], BIG1[29,34] and BIG2[35]) have an *in vitro* exchange activity that is sensitive to BFA. Surprisingly, however, BFA has little effect on the *in vitro* exchange activity of a subset of the Sec7-domain ARF GEFs (including ARNO[15], cytohesin-1[4], GRP1/ARNO3[37,38] and GBF1[31]).

The identification of BFA-sensitive and -insensitive ARF GEFs raises the question of what factors determine response to BFA. Studies both *in vitro* and *in vivo* pointed to the Sec7 domain itself as an important determinant. The isolated Sec7 domains of Sec7p[20,53] and BIG1[29] have an *in vitro* ARF exchange activity that is sensitive to BFA. Chimeric versions of Gea1p and Sec7p whose endogenous Sec7 domains had been replaced by that of the BFA-resistant ARNO Sec7 domain were resistant to BFA *in vivo* in yeast[54]. Random mutagenesis of the *GEA1* gene followed by selection of BFA-resistant clones identified a 35-amino-acid region of the Sec7 domain that is important for the effects of BFA (Fig. 2b). Strikingly, this region contains a high density of residues that differ between BFA-resistant and BFA-sensitive ARF GEF subfamilies but are highly conserved within each subfamily. Site-directed mutagenesis of a subset of these residues demonstrated their importance in determining BFA resistance[54,55].

The target of BFA is an ARF–GDP–Sec7-domain protein complex

Residues Y695 and M699 (in Gea1p) or F190 and M194 (in ARNO) affect BFA sensitivity *in vitro* and *in vivo* when mutated. These residues lie in the hydrophobic groove of the Sec7 domain, which forms the heart of the binding site for nucleotide-free ARF[19] (Fig. 2a). The simplest model to explain the mutagenesis data is that the hydrophobic BFA molecule binds to the hydrophobic groove of the Sec7 domain, thus preventing ARF binding. However, when this model was tested directly, Peyroche *et al.* found that, far from acting as a competitive inhibitor, BFA in fact acts as an uncompetitive inhibitor that stabilizes an ARF–GDP–Sec7-domain protein complex[54] (Fig. 4). Similar results were obtained for the Sec7 domain of human BIG1[29].

The hallmark of uncompetitive inhibition is that the target of the inhibitor is an enzyme–substrate complex rather than the enzyme itself. In the case of BFA, the target of the drug is a normally very-short-lived ARF–GDP–Sec7-domain reaction intermediate (Fig. 4). Remarkably, BFA dramatically stabilizes this normally transient intermediate in the exchange reaction, forming an ARF–GDP–BFA–Sec7-domain protein quaternary complex. Hence, BFA acts in a fashion analogous to a dominant–negative mutation.

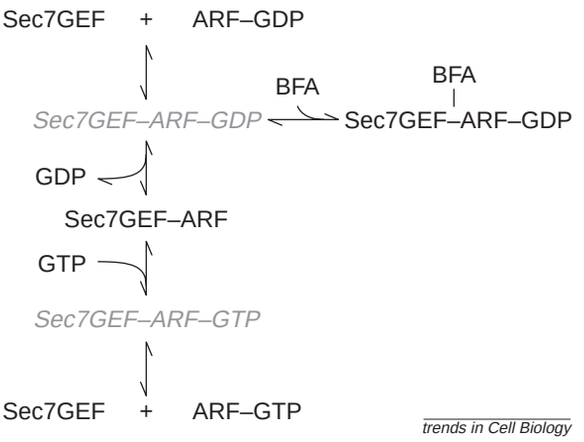

FIGURE 4

Mechanism of action of brefeldin A (BFA) on ADP-ribosylation factor (ARF). Brefeldin A binds to a normally very-short-lived ARF–GDP–Sec7-domain reaction intermediate and forms a stable quaternary complex, thus blocking the activation cycle of ARF. Unstable reaction intermediates are shown in italics.

This novel mechanism of action might serve as a paradigm for blocking other important signal-transduction pathways, such as the key pathways in the pathogenesis of cancer or other diseases. The concept of looking for small molecules that stabilize reaction intermediates (e.g. Ras–GEF or Rho–GEF complexes) could provide a novel strategy for the development of drugs useful for treating these diseases[56].

Are the Sec7-domain ARF exchange factors (or, more precisely, Sec7-domain–ARF complexes) the sole targets of BFA within the cell? In the secretory pathway of yeast, the Gea1p, Gea2p and Sec7p ARF GEFs are indeed the major targets of BFA as expression of BFA-resistant versions of these proteins renders yeast cells BFA resistant for both growth and secretion[54]. It remains to be seen whether a similar conclusion holds true for higher eukaryotes, but, in mammalian cells, BFA induces the ADP ribosylation of at least two proteins, one of which appears to function in Golgi membrane dynamics[57].

Conclusions and perspectives

In the three years since the first Sec7-domain ARF GEFs were identified, much progress has been made in characterizing their biochemical and molecular properties, but many important questions remain unanswered. The mechanisms by which the Sec7-domain proteins are localized to membranes are of crucial importance, because their location determines where ARF proteins will be activated in the cell. The most progress in this area has been made for the ARNO–cytohesin–GRP family members, which have a PH domain that preferentially binds to membranes containing PtdIns(3,4,5)P_3. For ARNO, PtdIns(4,5)P_2 is effective in recruiting the protein to membrane lipids *in vitro*, although it is an open question whether this effect is physiologically relevant. The large ARF GEFs do not have canonical PH domains,

and it is not known whether membrane recruitment occurs by interaction with specific types of lipids, a membrane-localized protein partner or both.

A second issue is the specificity of a given ARF GEF for a particular ARF protein *in vivo*. Some exchange factors (e.g. BIG1 and BIG2, which catalyse exchange on both class-I and -II ARFs, and ARNO, which can catalyse exchange on both ARF1 and ARF6) appear to be promiscuous *in vitro*, but is this also the case *in vivo*? Both *in vitro* and *in vivo* data indicate that a given ARF can have multiple activators, but the significance of this for ARF function in cells is not clear. Finally, no protein except ARF itself has been demonstrated to interact directly with any of the large Sec7-domain proteins, and few examples exist for the others. Sequence analysis alone does not shed much light on the question because the only recognizable motifs are the proline-rich domains of EFA6 and the coiled-coil motifs of EFA6 and the ARNO–cytohesin–GRP-family members.

Answers to the first two questions might well be obtained by identifying the interaction partners of Sec7-domain proteins. This avenue of research is sure to help us to understand the roles of this diverse family of proteins in membrane dynamics, protein transport and the integration of these processes into signal-transduction pathways within the cell.

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Differential sorting and post-secretory targeting of proteins in parasitic invasion

Huân M. Ngô, Heinrich C. Hoppe and Keith A. Joiner

Toxoplasma gondii uses a highly coordinated arsenal of three structurally and biochemically distinct secretory granules to invade and develop in a wide range of host cells. Proteins of these secretory granules are sorted to strategic subcellular locations using distinctive sorting signals and are then triggered differentially for exocytosis. These secreted proteins are subsequently targeted and inserted into membrane domains.

Apicomplexa are unicellular eukaryotes that are obligatory intracellular parasites with short-lived extracellular stages. The malarial agent *Plasmodium* spp. is the most 'potent' of this parasitic group of deadly pathogens of humans and livestock. *Toxoplasma gondii*, however, is the most widespread in host range and geographical distribution. Indeed, *T. gondii* can infect almost all nucleated cells and accounts for lifelong chronic infections in 10–90% of adult humans, depending on the geographical region. Such a successful professional invader must be capable of penetrating the host cell, eluding degradation by the host cell lysosomal pathway, establishing a safe haven where it can acquire nutrients from the host cell for rapid propagation, and then exiting the host cell. To achieve most of these objectives, apicomplexa have evolved an intricate and sophisticated network of constitutive and regulated secretory pathways that is unparalleled in most eukaryotic cells[1–6]. This review details the strategy used in *Toxoplasma*; a comparison with *Plasmodium* can be found elsewhere[1].

Spatial constraints of the cell

The *T. gondii* cell is relatively small (~2 × 8 μm), banana shaped and exhibits several striking features (Fig. 1). Most notable is the highly defined and stable cortical (membrane) skeleton, found in many protozoans[7], that limits the direct accessibility of vesicular traffic to the plasma membrane and extracellular environment. Underlying the plasma membrane is a tightly associated system of flattened cisternae and microtubules. Braced on the cytoplasmic face with a basket of 22 helical microtubules, the cortical cisternae (also known as the inner membrane complex) are sutured to form a quilt-like patchwork that continuously subtends the cell surface[8]. Consequently, accessibility to the plasma membrane is limited to the sutures between the cortical cisternae and to the apical (anterior) pole in the free parasite where the continuous cortical skeleton is interrupted at the apical ring of microtubules connecting to an extrusion known as the conoid[9].

Semipolarization of the secretory pathway

The limitation in surface accessibility might therefore impose a semipolarization of the *T. gondii* secretory pathway, which in turn provides directionality to the host cell invasion process that is driven by a cascade of exocytosis events initiated at the cell apical pole (see Figs 1 and 2). The nucleus is located near the centre of the cell, and the nuclear envelope is continuous with the endoplasmic reticulum (ER). Anterior to the nucleus is a single stack of

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