REVIEW

ArfGAPs: key regulators for receptor sorting

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Mammalian cells have many membranous organelles that require proper composition of proteins and lipids. Cargo sorting is a process required for transporting specific proteins and lipids to appropriate organelles, and if this process is disrupted, organelle function as well as cell function is disrupted. ArfGAP family proteins have been found to be critical for receptor sorting. In this review, we summarize our recent knowledge about the mechanism of cargo sorting that require function of ArfGAPs in promoting the formation of transport vesicles, and discuss the involvement of specific ArfGAPs for the sorting of a variety of receptors, such as MPR, EGFR, TfR, Glut4, TRAIL-R1/DR4, M$_5$-muscarinic receptor, c-KIT, rhodopsin and β1-integrin. Given the importance of many of these receptors to human disease, the studies of ArfGAPs may provide novel therapeutic strategies in addition to providing mechanistic insight of receptor sorting.

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Introduction

ArfGAPs are a protein family containing the ArfGAP domain. There are 31 genes encoding ArfGAPs in human[1]. ArfGAPs are structurally diverse, having a variety of domains other than the ArfGAP domain (Figure 1), consistent with the idea that each ArfGAP has a different function. They have the common function of catalyzing the hydrolysis of GTP that is bound to Arf, thereby converting Arf●GTP to Arf●GDP. The activity is essential for the function of Arf proteins, a family of proteins within the Ras superfamily of gunanine nucleotide binding proteins[2-3]. There are six Arfs (Arf1-Arf6) in mammals, though human lacks Arf2. Arf in GDP bound form is in cytosol with weak affinity for membrane. When GDP on Arf is converted to GTP by ArfGEF (Arf Guanine Nucleotide Exchange Factor) on the Golgi membrane, Arf●GTP binds to the Golgi membrane as well as coat protein, COPI. COPI is a coat protein that is recruited to the Golgi membrane by Arf●GTP and binds to cargos to produce COPI-coated vesicles from the Golgi apparatus[4]. Arf by itself cannot hydrolyze GTP, but the function of Arf requires GTP hydrolysis.

ArfGAP1 functions in COPI-mediated transport in pre-Golgi

The first insight into the role of ADP-ribosylation factor (Arf) and GTP hydrolysis in cargo sorting came up from the study of COPI-coated vesicle transport in pre-Golgi transport. Several groups reported that GTPγS, a non-hydrolyzable analogue of GTP and GTP-locked mutant of Arf (Arf1Q71L) blocked cargo incorporation into COPI vesicles, supporting the idea that GTP hydrolysis by Arf is critical for cargo sorting [5-8]. GTP bound to Arf is hydrolyzed by ArfGAPs (Arf GTPase activating protein), thereby converting Arf●GTP to Arf●GDP. ArfGAP1 is

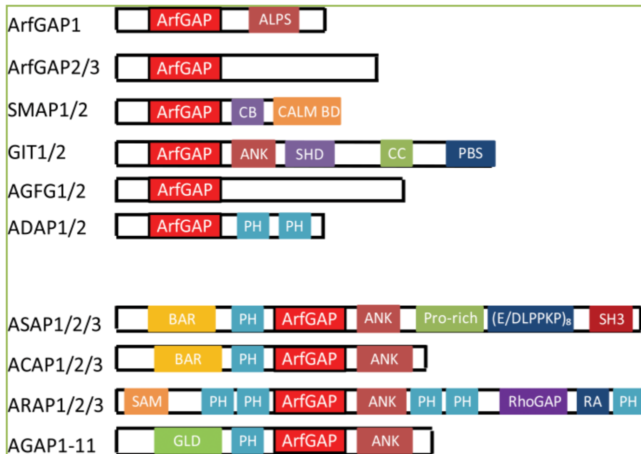

Figure 1. Schematic of domain structure of human ArfGAPs (modified from [15]). Not drawn to scale. Abbreviations are; ArfGAP, ArfGAP domain; ALPS, ArfGAP1 Lipid-Packing Sensor; CB, clathrin-box; CALM BD, CALM Binding Domain; ANK, ankyrin repeat; SHD, Spa-Homology Domain; CC, Coiled Coil; PBS, Paxillin Binding Site; PH, Preckstrin Homolody; $(E/DLPPKP)_8$, eight times repeat of E/DLPPKP sequence; SH3, Src-Homology 3; SAM, Sterile Alpha-Motif; RhoGAP, Rho Gap domain; RA, Ras-association domain; GLD, GTP-binding protein-Like Domain

functions of ArfGAPs, which are discussed in recent reviews[13-15].

Although most studies have focused on the role of Arf GAPs in uncoating transport vesicles, function in promoting the formation of coated transport vesicles has also been proposed. Initial evidence was generated using assays for in vitro generation of transport vesicles from purified Golgi membranes. Production of vesicles required functional ArfGAP1 able to hydrolyze GTP[12]. In cells, overexpression of ArfGAP1 increased the number of intracellular vesicular structures with the dimensions of COPI coated vesicles[16]. Overexpression of the GAP-dead mutant of ArfGAP1 (R50K) that still binds to Arf●GTP also results in producing vesicles. However, more STxB-KDEL, a COPI cargo, remained in the Golgi, implying that ArfGAP1(R50K) partially inhibits transport of a COPI cargo to the endoplasmic reticulum (ER)[16]. The concept that GAP activity of ArfGAP1 links vesicle production with cargo sorting is supported by the finding that peptide from the cytoplasmic tail of COPI cargos stimulates the GAP activity of ArfGAP1 in the presence of COPI in vitro[17]. Taking these results together, a model was proposed in which ArfGAP1 is required for the coupling of COPI and COPI cargo and promote COPI polymerization to produce COPI vesicles [15]. This model provides perspective for our discussion of the emerging roles of the function of ArfGAPs in receptor sorting in post-Golgi trafficking.

known to be involved in transport from the Golgi[9,10], therefore, ArfGAP1 has been thought to be a critical molecule to regulate cargo sorting in COPI transport[11,12]. ArfGAPs were also thought to be important for vesicle uncoating, which occurs after cargo sorting. Several models were proposed to explain the dual

ArfGAPs in Post-Golgi traffic

ArfGAP3 in MPR and EGFR traffic

Mannose 6-phosphate receptor (MPR) is the critical receptor required for the transport of lysosomal enzyme from the Golgi to the lysosome. A screen using siRNA targeting all ArfGAPs, in which effects on the localization of MPR were examined, identified ArfGAP3 as a regulator of transport of MPR from the early endosomes to the late endosomes[18]. ArfGAP3 had previously been reported to have a redundant role with ArfGAP1 and ArfGAP2 in COPI-mediated transport[19]. In contrast, ArfGAP3 effect on MPR traffic was found to be specific for ArfGAP3: ArfGAP1 or ArfGAP2 depletion did not affect the localization of MPR. Epidermal growth factor receptor (EGFR) transport from the early endosomes to the late endosomes was also perturbed in ArfGAP3 knock down cells whereas other transport pathways such as transferrin receptor recycling and Vesicular Stomatitis Virus Glycoprotein ts045 transport from the ER to the plasma membrane (PM) were not detectably affected. The results supported the idea that ArfGAP3 is specifically required for transport of MPR and EGFR from the early endosomes to the late endosomes. In the same paper, ArfGAP3 was reported to bind to Golgi-localizing, Gamma-adaptin ear domain, Arf-binding proteins (GGAs), which are coat proteins known to medicating transport of MPR. Therefore GGAs are likely the targets of ArfGAP3. These data support the idea that ArfGAP3 is required for promoting MPR transport by regulating GGAs. It was also discovered that ArfGAP3 regulates full-length MPR, but not luminal and transmembrane domain depleted MPR. It is possible that ArfGAP3 recognizes clustered MPR dependent on its luminal and transmembrane domain. Further study is required for revealing the mechanism by which ArfGAP3 regulates MPR sorting.

ArfGAP3 was reported to be an androgen target gene, and its overexpression promotes cell proliferation and migration of prostate cancer cells[20]. ArfGAP3 binds to Paxillin, an adaptor protein in focal adhesion and localizes to focal adhesions[20]. It will be interesting to examine whether ArfGAP3 regulates trafficking of integrin receoptors as well as EGFR and androgen receptor, which could account for its effects on migration and proliferation of the prostate cancer cells.

ACAP1 in tranferrin receptor, β1-integrin, and Glut4

recycling

ACAP family is the protein family that has BAR, PH, ArfGAP and ankyrin repeat domains. ACAP1 and ACAP2 have GAP activity towards Arf6 over Arf1 or Arf5 [21]. ACAP1 functions in cargo sorting by recognizing recycling cargos, such as transferrin receptor (TfR), β1-integrin and glucose transporter type 4 (Glut4), controlling translocation to the PM. ACAP1 binds to the cytoplasmic tail of TfR, and TfR recycling is slowed in ACAP1 knock down cells[22]. TfR is a marker for constitutive recycling pathway, however, ACAP1 also functions in regulated recycling of β1-integrin[23] and Glut4[24]. The recycling of β1-integrin to the PM upon serum stimulation is inhibited in ACAP1 knock down cells. ACAP1 binds to β1-integrin upon serum stimulation, and the binding is dependent on phosphorylation of ACAP1 by Akt. Depletion of ACAP1 or Akt inhibits cell migration[23]. The binding site between ACAP1 and β1-integrin was biochemically defined. The cytoplasmic tail of β1-integrin binds to C-terminal portion of ACAP1 that includes ArfGAP and ankyrin repeat domains [25]. Phosphorylation of ACAP1 enhances this binding. ACAP1 also seems to affect Glut4 transport to the PM under insulin stimulation in 3T3-L1 adipocytes, as ACAP1 depletion inhibits glucose uptake under insulin stimulation[24]. ACAP1 binds to clathrin, a major coat protein in post-Golgi, and showed colocalization with clathrin and Arf6 in Glut4 positive compartment. Depletion of clathrin and Arf6 also inhibits glucose uptake, therefore it is proposed that ACAP1 mediates recycling of Glut4 as a coat component of clathrin and Arf6.

ARAP1 in EGFR and DR4 traffic

ARAP1-3 are the family of proteins that have SAM, five PH, ArfGAP, ankyrin repeats, RhoGAP, and RA (Ras-associating) domains. ARAP1 (also known as Centaurin delta 2) has GAP activity towards Arf1 and Arf5 over Arf6 [26]. ARAP1 was reported to regulate EGFR transport [27-28]. There is a discrepancy between two reports. Yoon *et al*. reported that EGFR degradation is accelerated and phosphorylation of Extracellular- signal-Related Kinase (ERK) and c-Jun-N-terminal Kinase (JNK) is rapidly diminished in ARAP1 knock down cells. Daniele *et al*. reported that EGFR accumulated in sorting/late endosomes and EGFR degradation was slower in ARAP1 knock down cells compared to controls. The reason for this difference is unknown. In both reports, ARAP1 localized to the Golgi, an endosomal compartment and the PM, and internalization and recycling of transferrin were not perturbed by decreased expression of ARAP1. Kang *et al*. reported that ARAP1 binds to PTK6, a non-receptor tyrosine kinase and found that PTK6 binds and phosphorylates ARAP1 upon EGF stimulation [29]. The phosphorylation-defective mutant of ARAP1 accelerates EGFR degradation compared with wild type ARAP1, and silencing of PTK6 in breast cancer cells down-regulates EGFR. These results support the idea that ARAP1 inhibits EGF/EGFR degradation dependent on PTK6. One possibility is that ARAP1 promotes EGFR recycling to the PM upon EGF stimulation; therefore depletion of ARAP1 or expression of a phosphorylation-deficient mutant of ARAP1 accelerates EGF/EGFR degradation. Further studies are required for determining the mechanism by which ARAP1 specifically regulates EGFR upon EGF stimulation.

ARAP1 was also reported to regulate death receptor, TRAIL-R1/DR4[30]. ARAP1 binds to DR4 and TRAIL-R2/DR5 upon stimulation of apoptosis-inducing ligand, TRAIL, in human immortalized keratinocytes (NCTC). ARAP1 c-terminal region that lacks fifth PH domain (Δexon30) binds to DR4, and the presence of exon30 abolished the binding. This splicing variant of ARAP1 (Δexon30) is the predominantly expressed form in a wide variety of cell lines. The binding site on DR4 resides in the first two α-helices of the death domain of DR4. This region showed the highest binding to ARAP1 by co-immunoprecipitation. The knock down of ARAP1 results in the down-regulation of DR4 and slower initial phase of TRAIL-induced apoptosis. Total amount of DR4 is not changed upon ARAP1 knock down. It is possible that recycling of DR4 from an intracellular compartment to the PM is inhibited in ARAP1 knock down cells, similar to EGFR transport.

AGAP1 in M_5 muscarinic receptor traffic

AGAPs are the family of proteins that have GLD (GTP-binding protein-like domain), PH, ArfGAP and ankyrin repeats domains. There are 11 genes identified for AGAPs, however, AGAP4 to AGAP10 genes encode almost the same mRNA sequence[18], while the gene loci of these genes are different[1]. Probably these genes arose by gene duplication. AGAP1 (also known as centaurin gamma 2) has GAP activity towards Arf1 and Arf5 over Arf6[31], and was reported to regulate M_5 muscarinic receptor transport[32]. Muscarinic receptors (MRs) are a family of G-protein coupled receptors (GPCRs) for acetylcholine. Activation of MR5 (M_5) receptors potentiates dopamine release in neurons. Dysfunction of M_5 may contribute to the pathophysiology of schizophrenia and drug addiction. The precise function and regulation of M_5, particularly compared to other subtypes, has not been defined. Overall, there is significant sequence similarity among MR family members and the subtypes have not been found to have significant selectivity for MR ligands. Functional differences should be related to the large third intracellular loop (i3 loop) whose sequence is highly divergent between

subtypes. Bendor *et al*. found that AGAP1 is an interacting partner of the i3 loop of M_5 muscarinic receptor, and showed that the binding between AGAP1 and M_5 is specific among other MRs and AGAP family. M_5 mutant lacking AGAP1 binding site showed the inhibition of recycling of M_5 to the PM in cultured neurons, and the decrease of presynaptic M_5-mediated dopamine release potentiation in mice brain slices. AGAP1 has been reported to bind to AP-3[33], a coat-like adaptor complex that is known to be involved in the biogenesis of lysosomal related organelles such as synaptic vesicles[34]. M_5 was not detected in synaptic vesicles. Further studies are required for identifying the compartment from where M_5 is recycled to the PM. Both AP-3 and M_5 bind the PH domain of AGAP1, but it is not known whether the bindng sites are overlapping. It is possible that AGAP1 mediates complex formation of AP-3 and M_5. Whether the M_5 binding to PH domain affects the GAP activity of AGAP1 has yet to be studied. Further studies are required for understanding the mechanism by which AGAP1 regulates M_5 transport through AP-3.

AGAP2 in STxB transport

AGAP2 (also known as PIKE-A, Centaurin gamma 1 and GGAP2) has a very similar sequence as AGAP1 (71% identities in nucleotide level, 55% in amino acid) and has GAP activity towards Arf1 and Arf5 over Arf6 similar to AGAP1. However, its function seems to be different than AGAP1. AGAP2 depletion inhibits the retrograde transport of Shiga Toxin B subunit (STxB) from early endosomes to the trans-Golgi network (TGN) [35]. STxB binds to a glycosphingolipid, globotriaosylceramide (Gb3) [36] therefore AGAP2 regulates lipid-based transport. Gb3 does not have a cytoplasmic domain that can be recognized by cytosolic proteins. The mechanism how AGAP2 recognizes Gb3 is unknown.

AGAP2 was reported to bind to β-arrestins, which in turn bind to β2 adrenergic receptor (β2AR) and affects the intracellular localization of β2AR[37]. β-arrestins bind to PH domain of AGAP2. These results raise the possibility that PH domain of AGAP family is the binding site for cargos and the binding is coupled with regulation of GAP activity of AGAPs.

AGAP3 in AMPA receptor transport

AGAP3 (also known as centaurin gamma3, MRIP-1, and GGAP3) is another AGAP family protein whose amino acid sequence has 72% identity with AGAP1 and 52% identity with AGAP2. AGAP3 was reported to be a binding partner of SynGAP, a component of the N-methyl-D- aspartate receptor (NMDA) receptor complex [38]. NMDA receptor mediates long-term potentiation (LTP) in synapses. Following the activation of NMDA receptor, synaptic connection between two cells is strengthened by up-regulation of trafficking of AMPA-type glutamate receptors to the synapse. Downstream of the NMDA receptor, Ras/ERK signaling pathway is key to the induction of LTP, however, the link between Ras/ERK signaling and AMPA receptor trafficking is unknown. AGAP3 is co-precipitated with SynGAP and NR2A NMDA receptor complex. AGAP3 knock down increases the basal level of phospho-ERK signal, and decreases the phospho-ERK signal upon stimulation. AGAP3 binds to the GTP-locked mutant of Arf6, and AGAP3 overexpression decreases cellular Arf6-GTP levels. Whether AGAP3 has direct GAP activity towards Arf6 is unknown. AGAP3 overexpression also decreases Ras-GTP levels. Upon knock down of AGAP3, AMPA receptor on the cell surface in rat hippocampal cultures slightly increases (~20%), and this phenotype is rescued by GDP-locked mutant of Arf6. Upon stimulation, cell surface AMPA receptor decreases in AGAP3 knock down cells; in contrast, AMPA receptor increases in control cells. NMDA receptor transport is not affected by AGAP3 knock down. It is proposed that AGAP3 plays a role as a component of the NMDA receptor signaling complex that links activation of NMDA receptor to AMPA receptor trafficking.

ASAP1 in β1-integrin and rhodopsin transport

ASAP1 (also known as AMAP1, DDEF1, DEF1 or centaurin β4) is the most studied ArfGAP of the PH domain-containing ArfGAPs. ASAPs has BAR, PH, ArfGAP, ankyrin repeat, Proline-rich, (E/DLPPKP) repeat and SH3 domains. ASAP1 uses Arf1 and Arf5 as substrates in a 200-fold preference over Arf6[39]. ASAP1 localizes at the focal adhesions[40], and ASAP1 is implicated in tumor invasion and malignancy[41, 42]. Chromosomal amplification of ASAP1 has been found in uveal melanoma [41]. Overexpression of ASAP1 was found in prostate cancer[43], ovarian cancer[44], breast cancer[45], hepatocellular carcinoma[46], and colorectal cancer[47]. ASAP1 binds to a number of signaling proteins including PRKD2[39, 48-55]. PRKD2 binds to β1-integrin, and ASAP1 or PRKD2 knock down inhibits the recycling of β1-integrin upon EGF stimulation in breast cancer cells[54]. Overexpression of a fragment of PRKD2 that inhibits endogenous binding between ASAP1 and PRKD2 also inhibits β1-integrin recycling upon EGF stimulation, therefore the binding between ASAP1 and PRKD2 is required for β1-integrin recycling. The depletion of ASAP1 or PRKD2, or overexpression of a fragment of PRKD2 that inhibits the binding between ASAP1 and PRKD2, inhibits matrigel invasion but not adhesion to collagen of breast cancer cells.

ASAP1 is proposed to be an effecter of Arf6[42, 56], as

ASAP1 binds to GTP-locked mutant of Arf6 over Arf1 and Arf5[57]; however, the interaction does not appear to be direct. Inoue *et al*. reported that ASAP1 binds to the Arf6/Rab11 binding proteins Fip3 and Fip4, and that there is a likely indirect interaction with Arf6 mediated by Fip3/4[52]. As ASAP1 GAP activity is towards Arf1 and Arf5 in vitro[39], and ASAP1 knock down leads to up-regulation of Arf1□GTP levels in fibroblast cells[58], the direct substrate of ASAP1 is likely Arf1. It is possible that there is a signaling cascade such as GTP hydrolysis on Arf1 by ASAP1 occurs in the downstream of Arf6 binding to ASAP1.

ASAP1 has also been reported to function in ciliary transport of rhodopsin in photoreceptor cells and polycystin-1 in retinal cortical tubular epithelia (RCTE) and Madin-Darby canine kidney (MDCK) cells[59-61]. The VxPx motif in the cytoplasmice tail of rhodoposin binds to Arf4[62], and makes a complex with ASAP1. Depletion of ASAP1 or inhibition of GAP activity of ASAP1 inhibits incorporation of rhodopsin into rhodopsin-transport carriers[60], and ciliary targeting of rhodopsin[61]. ASAP1 also binds to another ciliary targeting motif, the FR motif in cytoplasmic tail of rhodoposin separately from Arf4. Wang *et al*. proposed that ASAP1 serves as a platform for Rab11- FIP3 and Rabin8-Rab8 complexes sequentially. Another ciliary targeting cargo, polycystin-1 also has the VxPx motif, and binds to Arf4 and ASAP1[59].

SMAP1 in c-KIT transport

SMAP1/2 are ArfGAPs with a clathrin-binding motif, and their overexpression inhibits clathrin dependent transport [63-65]. SMAP1 has GAP activity towards Arf6[63], and SMAP2 towards Arf1 and Arf6 [64]. C-KIT(CD117) is a surface marker for hematopoietic progenitor cells. C-KIT is a receptor tyrosine kinase and its ligand is stem cell factor (SCF). In SMAP1 knock out mouse, c-KIT degradation upon SCF stimulation is inhibited in bone marrow-derived mast cells (BMMCs) and transport of c-KIT to the lysosome is inhibited in mouse embryo fibroblast (MEF) cells[66]. In *SMAP1*$^{-/-}$ MEF cells, c-KIT colocalizes with multi-vesicular bodies (MVBs) markers such as Hrs and Rab7, therefore it is thought that SMAP1 plays a role in transport of c-KIT from MVBs to the lysosome. Interestingly, EGFR transport is not affected in *SMAP1*$^{-/-}$ MEF cells, suggesting the function of SMAP1 in c-KIT transport to the lysosome is specific. This result is consistent the general model that we have proposed that ArfGAPs are cargo–specific regulators of membrane traffic.

Concluding remarks

The role of ArfGAPs in post-Golgi traffic is just beginning to emerge. Recent work indicates that ArfGAPs are necessary for specific intracellular trafficking of receptors. The specificity between cargos and ArfGAPs, and the relationship between cargo recognition and regulation of GAP activity, are still poorly understood. The spatial, temporal, and molecular level studies are required for understanding these processes. These studies will reveal the importance of ArfGAPs in physiology and disease states that lacks proper regulation of intracellular trafficking.

Conflicting interests

The authors have declared that no competing interests exist.

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