Substrate specificities and activities of AZAP family Arf GAPs in vivo

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Cuthbert EJ, Davis KK, Casanova JE. Substrate specificities and activities of AZAP family Arf GAPs in vivo. Am J Physiol Cell Physiol 294: C263–C270, 2008. First published November 14, 2007; doi:10.1152/ajpcell.00292.2007.—The ADP-ribosylation factors (Arfs) are a family of small GTPases that are key regulators of vesicular transport in eukaryotic cells. Like other GTPases, the Arfs require guanine nucleotide exchange factors to facilitate GTP loading and GAP-activating proteins (GAPs) to promote GTP hydrolysis. Whereas there are only six mammalian Arfs, the human genome encodes over 20 proteins containing Arf GAP domains. A subset of these, referred to as AZAPs (Randazzo PA, Hirsch DS. Cell Signal 16: 401–413, 2004), are characterized by the presence of at least one NH2-terminal pleckstrin homology domain and two or more ankyrin repeats following the GAP domain. The substrate specificities of these proteins have been previously characterized using in vitro assay systems. However, a limitation of such assays is that they may not accurately represent intracellular conditions, including posttranslational modifications, or subcellular compartmentalization. Here we present a systematic analysis of the GAP activity of seven AZAPs in vivo, using an assay for measurement of cellular Arf-GTP (Santy LC, Casanova JE. J Cell Biol 154: 599–610, 2001). In agreement with previous in vitro results, we found that ACAP1 and ACAP2 have robust, constitutive Arf6 GAP activity in vivo, with little activity toward Arf1. In contrast, although ARAP1 was initially reported to be an Arf1 GAP, we found that it acts primarily on Arf6 in vivo. Moreover, this activity appears to be regulated through a mechanism involving the NH2-terminal sterile-α motif. AGAP1 is unique among the AZAPs in its specificity for Arf1, and this activity is dependent on its NH2-terminal GTPase-like domain. Finally, we found that expression of AGAP1 induces a surprising reciprocal activation of Arf6, which suggests that regulatory crosstalk exists among Arf isoforms.

THE ADP-RIBOSYLATION FACTORS (Arfs) are a family of small GTPases that are key regulators of vesicular transport in eukaryotic cells. The six mammalian Arfs are divided into three classes on the basis of sequence relatedness; Arfs 1, 2, and 3 in class I; Arfs 4 and 5 in class II; and Arf6 in class III. Whereas Arf1 is thought to regulate trafficking within and from the Golgi, Arf6 functions primarily within the plasma membrane-endoosomal system, where it has been implicated in many aspects of plasma membrane trafficking: endocytosis, regulated exocytosis, and postendocytic recycling (9).

Like most small GTPases, Arfs cycle between a membrane-localized, GTP-bound “active” state and a cytosolic GDP-bound “inactive” state. In the active conformation, Arfs interact with multiple downstream effector proteins that mediate their ability to control vesicular transport. These effectors include components of a variety of vesicle coat complexes, including COP1, AP-1, AP-3, and the monomeric GGA adapter proteins, as well as phospholipid-modifying enzymes such as phospholipase D, phosphatidylinositol 4-kinase, and phosphatidylinositol (4)P-5-kinase (2–4, 29). As for most other GTPases, completion of the GTPase cycle requires the function of guanine nucleotide exchange factors to catalyze loading with GTP, and GTPase-activating proteins (GAPs) to stimulate GTP hydrolysis. Over 20 genes encoding proteins with a recognizable Arf GAP domain are present in the human genome (26). In addition to their GAP domains, many of these proteins also contain multiple binding motifs that allow them to interact with phospholipids and other protein components of the signaling and transport machinery (11, 26).

The AZAPs constitute a family of Arf GAPs that are characterized by an NH2-terminal pleckstrin homology (PH) domain and a central Arf GAP domain followed by two or more ankyrin repeats. On the basis of sequence and domain organization, the AZAP family is further subdivided into four subfamilies: 1) the ACAPs contain an NH2-terminal bin/amphiphrin/Rvs (BAR) domain (a phospholipid-binding domain that is thought to sense membrane curvature), a single PH domain followed by the GAP domain, and four ankyrin repeats; 2) the ASAPs also contain an NH2-terminal BAR domain, the tandem PH domain/GAP domain, three ankyrin repeats, two proline-rich regions, and a COOH-terminal Src homology 3 domain; 3) the AGAPs contain an NH2-terminal GTPase-like domain (GLD), a split PH domain, and the GAP domain followed by four ankyrin repeats; and 4) the ARAPs contain both an Arf GAP domain and a Rho GAP domain, as well as an NH2-terminal sterile-α motif (SAM), a proline-rich region, a GTPase-binding domain, and five PH domains (26). The distinctive domain organization of each subfamily is shown in Figure 1.

GAP activity has previously been demonstrated for each of these proteins in in vitro assays by using purified Arfs, GAPs, and phospholipids. Results from such assays have suggested that ACAP1 and ACAP2 prefer Arf6 as a substrate (13) and that AGAP1 prefers Arf1 (23). In most cases, GAP activity is stimulated by the presence of phosphoinositides and phosphatidic acid. Whether these lipids serve to recruit the GAP to the surface of lipid micelles or liposomes, to allosterically modulate catalytic activity, or both remains to be determined.

As a group, the AZAPs are large proteins, and it has proven difficult to produce many of them in full-length recombinant form. For this reason, many of the published reports have measured GAP activity by using truncated forms that may not accurately reflect the activity of the full-length protein, or immunoprecipitates of full-length epitope-tagged proteins from cell lysates. Whereas immunoprecipitates may allow for posttranslational modifications that can occur only in eukary-
otic cells, they may also contain additional coprecipitating proteins that could affect activity. In addition, a significant limitation of such assays is that all spatial information is lost; proteins that may serve as substrates in vitro may never encounter a specific GAP in vivo if they are present in distinct cellular compartments. Some investigators have attempted to assign substrate specificity on the basis of phenotypes of GAP-transfected cells that mimic those of dominant-negative Arf expression; however, such reports have produced conflicting results (15, 30). For these reasons, the in vivo substrate specificity of each AZAP remains poorly defined. Our laboratory has developed a pull-down assay that allows the measurement of Arf activation in intact cells (27). Using this assay, we have characterized the GAP activity of seven AZAP family members in vivo. Although the substrate specificity of several AZAPs is similar to the previously reported in vitro assays, we found that several proteins had no detectable activity in intact cells and that ARAP1 had very different substrate specificity than was previously reported. Moreover, ARAP1 activity appears to be regulated by its NH2-terminal domain. In addition, we found that expression of AGAP1, which promotes down-regulation of Arf1 activity, actually enhanced the activation of Arf6, which suggests that cross talk may exist among Arf regulation of Arf1 activity, actually enhanced the activation of Arf6, which suggests that cross talk may exist among Arf family members in vivo.

**MATERIALS AND METHODS**

**Reagents, Antibodies, and DNA Constructs**

**Antibodies.** Monoclonal anti-FLAG antibody, M2, was from Sigma (St. Louis, MO), and monoclonal anti-HA antibody (16B12) was from Covance (Berkeley, CA). Secondary antibodies, Cy2-donkey anti-mouse, Cy2-donkey anti-rabbit, Texas red goat anti-mouse, and Texas red donkey anti-rabbit, were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase-coupled sheep anti-mouse and goat anti-rabbit secondary antibodies from Amersham Biosciences (Piscataway, NJ) were used for Western blot analysis.

**Plasmids.** FLAG-tagged AZAP constructs were provided by Paul Randazzo (National Cancer Institute, Bethesda, MD) and have been previously described (12, 13, 21, 23). Green fluorescent protein (GFP)-ARAP3 constructs were obtained from Sonia Krugmann (Babraham Research Institute, Cambridge, UK) and have been previously described (16).

**Cell Culture and Transfections**

Baby hamster kidney cells were maintained in complete DMEM (GIBCO) supplemented with 10% fetal bovine serum and antibiotics. HeLa cells were grown in high-glucose DMEM. Cells were transfected with DNA plasmids by using Fugene as recommended by the manufacturer (Roche).

**Immunofluorescence Microscopy**

Cells cultured on glass coverslips were transfected as described above. Twenty-four hours later, the cells were fixed in 4% paraformaldehyde, then permeabilized in PBS containing 0.2% saponin, and blocked by using 10% normal goat serum. Cells were then incubated with M2 anti-FLAG monoclonal antibody to detect exogenous GAPs, followed by Cy2-conjugated donkey anti-mouse. In cotransfected cells, Arf expression was imaged by using rabbit polyclonal antibodies raised against Arf1 or Arf6, followed by Cy3-conjugated donkey anti-rabbit antibody. Cells were imaged on a Nikon C1Plus confocal microscope with a ×60, 1.4 numerical aperture objective. Images shown have not been processed to enhance brightness or contrast.

**Measurement of Arf-GTP**

Baby hamster kidney cells were cotransfected with HA-tagged Arf1 or Arf6 and either empty vector or plasmid encoding individual FLAG-tagged or GFP-tagged AZAPs (at a 1:4 ratio of Arf:AZAP). Immunofluorescence microscopy confirmed that >90% of transfected cells expressed both Arf and the cotransfected GAP. Arf activation was assayed by using a previously described pull-down assay in which Arf6-GTP is recovered from cell lysates on the basis of its affinity for the Arf-binding domain of the adaptor protein GGA3 [2].

Proteins bound to GST-GGA3 (40 μg) were eluted into SDS-PAGE sample buffer and were quantitated by Western blotting with a monoclonal anti-HA antibody (16B12). Total levels of each Arf in the starting lysates were assayed by immunoblotting of 5% of the clarified lysate. Bands were quantitated by densitometry, and the ratio of Arf-GTP to total Arf was calculated for each condition. In each experiment, the ratio obtained from control cells (expressing Arf alone) was set to a value of 1. Immunoblotting of total cell lysates was also used to confirm equivalent levels of expression for each GAP construct. All experiments were performed at least three times, and the graphs represent the mean ± SD for each condition.

**RESULTS**

The domain organization of the four AZAP subfamilies is shown in Fig. 1. Here we examined the activities of two representatives of the ACAP subfamily (ACAP1 and ACAP2), one ASAP (ASAP1), two AGAPs (AGAP1 and AGAP2), and two ARAPs (ARAP1 and ARAP3). To facilitate detection in transfected cells, all constructs were FLAG tagged at their NH2 termini, except ARAP3, which was GFP tagged. As shown in Fig. 1, immunofluorescence microscopy revealed that the transfection efficiency of each construct was roughly equivalent, that the levels of expression were moderate, and also comparable to each other. Equivalent levels of expression were also confirmed by immunoblotting (data not shown). As expected, differences were observed in the subcellular localization of the different AZAPs. As previously reported for ACAP1 (8, 13, 18), ACAP1 and ACAP2 displayed a primarily intracellular distribution on short tubular structures. The distribution of ASAP1, ARAP1, and ARAP3 was also primarily intracellular but more diffuse. In contrast, AGAP2 was concentrated in discrete intracellular puncta scattered throughout the cytoplasm, presumably endosomal in nature. Finally, AGAP1 appeared to localize primarily to the plasma membrane, where it was concentrated in membranous protrusions.

To characterize the activities of individual AZAP family members in intact cells, we used a well-established Arf-GTP pull-down assay developed in our laboratory (27). Briefly, cells expressing low levels of either Arf1-HA or Arf6-HA were cotransfected with individual Arf GAP constructs, and GTP-bound Arf was selectively precipitated via its interaction with the Arf-binding domain of the adaptor protein GGA3. Arf-GTP levels were quantitated by immunoblotting and were normalized to total cellular Arf1 or Arf6. For each Arf GAP construct tested, the basal level of Arf activity was determined in cells expressing Arf1-HA or Arf6-HA alone, and this value was set to 1. Levels of Arf-GTP in cells coexpressing each GAP were then expressed as a percentage of the control value. Constructs encoding ACAP1, ACAP2, ASAP1, AGAP1, AGAP2, and ARAP1 were all FLAG tagged to allow direct comparison of expression levels in transfected cells. For each GAP, a corresponding catalytically inactive mutant containing a conservative point mutation in the catalytic domain was tested in parallel as a negative control.
AGAP1 is an Arf1 GAP in Vivo

We first examined the ability of each AZAP to catalyze GTP hydrolysis on Arf1. Among this Arf GAP family, ASAP1, AGAP1, AGAP2, and ARAP1 have been reported to act as Arf1 GAPs in vitro (10, 13, 20–22). Surprisingly, we found that, of the seven Arf GAPs tested, only AGAP1 significantly reduced the level of Arf1-GTP in expressing cells (Fig. 2). Expression of AGAP1 decreased the levels of Arf1-GTP by 42% of control cells, while the catalytically inactive mutant AGAP1(R599K) had no obvious effect. The residual Arf1 activity observed in cells expressing wild-type AGAP1 was visualized by incubation with M2 monoclonal FLAG antibody.

Fig. 1. Domain organization and localization of the four AZAP families. BAR: bin/amphiphysin/Rvs domain; PH: pleckstrin homology domain; GAP: ADP-ribosylation factor (Arf) GTPase-activating protein domain; A: ankyrin repeat; SH3: Src homology domain 3; GLD: GTPase-like domain; SAM: sterile-α motif; PR: proline-rich domain; RA: Ras-association domain. HeLa cells were transfected with wild-type (WT) FLAG-tagged AZAP constructs or green fluorescent protein (GFP)-tagged ARAP3 as indicated. Cells were fixed and permeabilized, and the FLAG-tagged AZAP was visualized by incubation with M2 monoclonal FLAG antibody.

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AGAP1 is likely due to the large pool of active Arf1 in the Golgi that is regulated by GAPs other than AGAP1.

A smaller decrease in Arf1-GTP (34%) was also observed in cells expressing ACAP1 (Fig. 2). Interestingly, the catalytically inactive ACAP1 mutant ACAP1(R448Q) induced a 64% increase in Arf1-GTP levels. This could be because the catalytically inactive mutant acts in a dominant-negative manner to repress the function of the endogenous protein. Alternatively, this could be an indirect effect of the failure of ACAP1 to act on another Arf (see below).

As mentioned above, ARAP1 was previously reported to have Arf1 GAP activity in vitro (21). However, we found that ARAP1 not only failed to reduce Arf1-GTP levels, it actually enhanced Arf1 activation by more than twofold. A similar, but less pronounced, activation of Arf1 was observed in the presence of the related protein ARAP3. Although we were unable to generate a catalytically inactive mutant of ARAP1, the corresponding ARAP3 mutant did not increase Arf1-GTP levels, which suggests that the observed change is a result of GAP activity, possibly on another Arf.

Both ACAP1 and ACAP2 Function as Arf6 GAPs in Vivo

We next examined the effects of each of the AZAPs on Arf6-GTP levels in intact cells. As shown in Fig. 3, both ACAP1 and ACAP2 exhibited significant activity on Arf6, with Arf6-GTP levels reduced by 81% for ACAP1 and 85% for ACAP2, relative to controls. These data are in agreement with previously reported in vitro assays that show a clear preference towards ARAP3, these observations suggest that cross talk may exist between Arf family members.

The ARAP1 NH2 Terminus Negatively Regulates Arf6 GAP Activity

Database analysis indicates that ARAP1 exists in multiple splice isoforms, the longest of which (isoform C) contains an NH2-terminal SAM and adjacent proline-rich region that is present in the other ARAP family members (12, 21). The two shorter isoforms (isoforms A and B) appear to use alternative start sites, resulting in loss of the SAM and proline-rich domains. SAM domains have been shown to mediate homodimerization in other proteins and may also mediate heterologous protein-protein interactions (14). Since the absence of the NH2 terminus could affect activity, we compared the Arf GAP activity of isoform A, which we call ARAP1(ΔSAM), to that of the longer isoform (isoform C). The two isoforms exhibited similar subcellular distributions, as shown by immunofluorescence microscopy (Fig. 4B).

As shown in Fig. 2, the long isoform actually stimulated Arf1 activation relative to controls. ARAP1(ΔSAM) had a similar, but less pronounced, stimulatory effect (1.5-fold vs. 2-fold; Fig. 4C). Intriguingly, expression of an ARAP1 mutant lacking Arf GAP activity, ARAP1(ΔSAM)R338K, enhanced Arf1 activation to a similar extent, suggesting that this effect does not require Arf GAP activity and may therefore be indirect. In agreement with this hypothesis, an ARAP1 mutant lacking Rho GAP activity [ARAP1(ΔSAM)R753K] failed to stimulate Arf1 activation, suggesting that downregulation of RhoA activity may lead to enhanced activation of Arf1.
As noted above, the long isoform of ARAP1 neither stimulated nor inhibited Arf6 activation relative to controls. In contrast, we found that expression of ARAP1(SAM) reduced Arf6-GTP levels by nearly 75% (i.e., similar to ACAP1), and this was clearly dependent on Arf GAP activity (Fig. 4C). Moreover, unlike Arf1, the activation of Arf6 and RhoA appears to be functionally uncoupled, since mutation of the Rho GAP domain had no effect on Arf6 activation. Together, these results suggest that ARAP1 functions as an Arf6 GAP in vivo, in contrast with the previously reported Arf1 specificity defined in vitro. In addition, the significantly lower activity of the full-length protein relative to that of the shorter SAM isoform suggests that this catalytic activity is negatively regulated by the NH2-terminal domain.

AGAP1 Enhances Arf6 Activity

As shown in Fig. 3, we unexpectedly found that while expression of AGAP1 reduced the levels of Arf1-GTP, it also resulted in a significant increase in the levels of Arf6-GTP. Expression of the catalytically inactive mutant AGAP1(R599K) had an even more pronounced effect on Arf6-GTP levels, ranging from 2.5- to 8-fold higher than in cells expressing Arf6 alone. Curiously, this effect was specific to Arf6, because expression of AGAP1R599K had no effect on Arf1-GTP levels. This suggests that the GAP domain of AGAP1 acts on Arf1, whereas another domain may mediate the enhancement of Arf6 activity.

As described in the introduction, the AGAPs are characterized by the presence of an NH2-terminal GLD that could fulfill this function. To test this hypothesis, we coexpressed an AGAP1 mutant lacking the GLD, AGAP1(GLD), with either Arf1 or Arf6. Despite similar expression levels (Fig. 5B), we found that this mutant lost its ability to stimulate GTP hydrolysis on Arf1 (Fig. 5C). Coincident with this, AGAP1(GLD) also lost its ability to stimulate Arf6 activation. Even more strikingly, the corresponding catalytically inactive mutant, AGAP1(GLD)R599K, also failed to activate Arf6. Together, these observations suggest that the NH2-terminal GLD is...
required for both Arf1 GAP activity and the corresponding enhancement of Arf6-GTP levels. We hypothesized that the GLD domain may be important for localizing the protein to the proper site of activation. In support of this hypothesis, Fig. 5B shows that both the ΔGLD and ΔGLD R599K mutants failed to accumulate in the membranous protrusions at the plasma membrane where wild-type AGAP1 is found. These data suggest that proper localization of AGAP1 is important for both its Arf1 GAP activity and its role in Arf6 activation.

DISCUSSION

As a family, members of the AZAP class of Arf GAPs are characterized by their relatively large size and the presence of multiple noncatalytic domains that mediate their interaction with other proteins and with phospholipids. Although in vitro assays have provided insight into the potential substrate specificity and regulation of many of these proteins, such assays are limited in that they may not accurately reflect intracellular conditions. In particular, subcellular compartmentalization cannot be mimicked in vitro and all spatial information is lost.

Here we employed an assay that allows us to measure the activity of each GAP in the context of the intact cell. In some instances, our data have confirmed previous results from in vitro assays. For example, we determined that both ACAP1 and ACAP2 are robust, constitutive Arf6 GAPs, whereas AGAP1 appears to act preferentially on Arf1. However, we also found that ASAP1 and AGAP2, which have been shown to have GAP activity in vitro, did not exhibit detectable activity in intact cells. Possible explanations for this observation are...
discussed below. In addition, we found that the substrate specificity of at least one GAP, ARAP1, was radically different from its previously reported in vitro specificity and that two splice isoforms of ARAP1 that differ at their NH\textsubscript{2}-termini have dramatically different levels of activity in intact cells. Finally, our results revealed a surprising level of cross talk between Arf1 and Arf6 that may be mediated by Arf GAPs.

\textit{ASAP1} and \textit{AGAP2}. One surprising outcome of our studies is the observation that neither ASAP1 nor AGAP2 exhibited detectable GAP activity in vivo. Both proteins have been shown to efficiently catalyze GTP hydrolysis on Arf1 and Arf5 in vitro (6, 12, 13, 22). There are several possible explanations for this discrepancy. First, both proteins may become spatially segregated from Arf1 in intact cells, and it is possible that Arf5 is their primary substrate in vivo. For technical reasons, we have been unable to coexpress Arf5 with any of the AZAPs, and we have therefore been unable to definitively determine whether this is the case. Second, the activity of these proteins may be acutely regulated by signaling pathways whose activity is low under basal conditions. Stimulation of cells with growth factors, hormones, or other extracellular cues may be necessary to trigger their activation. A third possibility is that ASAP1 and/or AGAP2 may act on a relatively small pool of Arf1 in vivo. For example, ASAP1 has been reported to localize to focal adhesions (19, 25), a region of the cell that is relatively deenriched in Arf1. In this instance, the high background level of Arf1-GTP that derives from the Golgi could obscure relatively small changes in the active pool that occur in the cell periphery.

\textit{ARAPs}. The three ARAP proteins are characterized by the presence of both Arf GAP and Rho GAP domains and five PH domains. Previous reports have suggested that the short isoform of ARAP1 prefers Arf1 as its substrate in vitro (21), whereas ARAP3 has been reported to prefer either Arf5 (12) or Arf6 (16). We were therefore surprised to discover that neither ARAP1 nor ARAP3 exhibited measurable GAP activity at steady state when expressed in cells.

Database analysis indicates that all three ARAPs exist in multiple splice isoforms that differ in the presence or absence of an NH\textsubscript{2}-terminal SAM domain (12). SAM domains have been shown to promote homodimerization in other proteins and may also mediate heterologous protein-protein interactions (14). In comparing the two isoforms of ARAP1, we found that, in contrast with the long form, the shorter form lacking the SAM domain (isoform A) displayed robust constitutive GAP activity. However, this activity was not directed at Arf1 as predicted from previous in vitro assays (23), we found that the deletion of this domain completely abrogated activity in our in vivo assay. Together, these observations suggest that the GLD is not involved in catalysis but may be required for appropriate subcellular localization. No specific function has yet been assigned to the GLD; however, several reports have suggested that it does bind GTP (1, 28, 32, 33). A related domain found on Eps15 homology domain-containing protein 1 is required for membrane association (7, 17), and it is possible that the AGAP1 GLD has a similar role. Fig. 5B shows the absence of the two AGD mutants from the membranous protrusions where wild-type AGAP1 is found, supporting such a role for the GLD domain. Further work will be required to test this hypothesis.

\textit{Cross talk between Arf isoforms}. A particularly surprising outcome of these studies is the degree to which the activation states of Arf1 and Arf6 are interrelated. This was particularly apparent in the case of AGAP1, whose expression led to reduced Arf1 activity and a corresponding increase in Arf6 activation. How may such reciprocal regulation be achieved? One possible explanation is that Arf1 and Arf6 may regulate distinct transport pathways emanating from the same endosomal compartment and that inhibiting one pathway leads to reciprocal upregulation of the other. Such a scenario could operate in perinuclear recycling endosomes, where transferrin receptor recycling is thought to require Arf1 (31), whereas recycling of β1 integrins and class I major histocompatibility complex requires Arf6 (5). Inhibition of the Arf1-mediated pathway by expression of either wild-type AGAP1 (which reduces Arf1-GTP levels) or catalytically inactive AGAP1 (which presumably impairs Arf1 cycling) could cause a compensatory increase in membrane flow through the Arf6-mediated pathway and a corresponding increase in Arf6 activation. In agreement with this hypothesis, we observed a similar, but less pronounced, reciprocal activation of Arf1 in cells expressing a catalytically inactive mutant of the Arf6 GAP, ACAP1 (Fig. 2). Alternatively, AGAP1 could promote Arf6 activation in a manner that is independent of its GAP domain, by promoting the recruitment of an Arf6 guanine nucleotide exchange factor or inhibiting the activity of an Arf6-specific...
GAP. Further experimentation will be necessary to resolve this issue. Together, these observations highlight the importance of cellular context in defining the function of complex regulatory molecules such as the AZAPs. It will be interesting in the future to determine how interaction of these proteins with specific binding partners may modulate their activity. It will also be important to determine which, if any, of the AZAPs acts on Arf4 or Arf5, and to define the roles of such GAPs in vesicular transport.

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REFERENCES