The role of ArfGAP1 as a terminator or effector in COP-I-vesicle formation has been the subject of ongoing discussions. Here, the discussion on the putative terminator/effector functions has been enlarged to include Arf GAP members involved in the formation of clathrin-coated vesicles. ACAP1, whose role has been studied extensively, enhances the recycling of endocytosed proteins to the plasma membrane. Importantly, this positive role appears to be an overall reflection of both the terminator and effector activities attributed to ACAP1. Other Arf GAP subtypes have also been suggested to possess both terminator and effector activities. Interestingly, while most Arf GAP proteins regulate membrane trafficking by acting as facilitators, a few Arf GAP subtypes act as inhibitors.

ArfGAP1 is a prototype of Arf GAP family proteins, and is involved in COPI-coated vesicle formation. In the preceding Blog published in the previous issue of Cellular Logistics, R. Kahn sparked a debate on whether ArfGAP1 functions as a terminator of Arf signaling, as an Arf-dependent effector, or as both.1 This discussion was followed by V. Hsu and R. Beck et al. who focused on the effector and terminator functions of ArfGAP1.2,3 N. Segev then suggested experiments that might help shed light on this issue.4 In the above discussions, a terminator function refers to an ArfGAP1 interaction with GTP-bound Arf that results in the hydrolysis of GTP and the inhibition of a biological response, whereas an effector function refers to an ArfGAP1 interaction with GTP-bound Arf that results in the stimulation of a biological response, independently of GAP activity.

We would like to extend this viewpoint to the biogenesis of clathrin-coated vesicles in which many Arf GAP proteins are involved (see Table 1 for each Arf GAP subtype). This review will investigate the possibility that there is an analogy between a role for ArfGAP1 in COPI-vesicle formation and that of Arf GAP protein in clathrin-vesicle formation. However, compared to ArfGAP1, what is known for clathrin-related Arf GAPs appears very limited, if the above definition of terminator/effector is strictly applied.

Therefore, we have designated Arf GAP as a putative "terminator" if its GAP activity converts Arf to its GDP-bound form. The terminator function is principally dependent on the existence of the Arf GAP domain, and evidence for such a function would be forthcoming if the following experiments were carried out. Firstly, protein preparations of Arf GAP and its GAP-negative version could be compared through in vitro vesicle formation reconstitution assays. Secondly, wild-type and GAP-negative Arf GAP expression vectors could be transfected into tissue culture cells and their activities compared.

We tentatively utilize the term "effector" if Arf GAP exerts its activity through protein-protein interactions rather than through GAP activity. This activity would be mediated through various domains or motifs in Arf GAP and the interacting proteins would encompass a coat protein, an adaptor or the cargo itself, except for interacting molecules not involved in vesicle formation. Arf GAP protein interactions might be Arf-related, but Arf GAPs might also have Arf-independent functions. The putative effector function of Arf GAPs could be demonstrated experimentally as follows. First is colocalization of Arf GAP and its interacting proteins on the membrane of an organelle of interest. Second is co-immunoprecipitation from cell lysates and/or GST-pull down assay using cell lysates or purified proteins to identify the physical association of proteins. Third is expression and assessment of wild-type and mutant Arf GAP incapable of interacting with an effector molecule in tissue culture cells.

In another experimental approach, namely the knockdown of endogenous Arf GAP, certain caveats need to be taken into consideration. Arf GAP knockdown is achieved by introducing a specific siRNA into tissue culture cells or by gene targeting in mice. Inhibition of membrane trafficking as a consequence of Arf GAP knockdown would suggest that Arf GAP plays a positive role in the respective transport pathway. Conversely, if knockdown results in an enhancement of trafficking, then the particular Arf GAP can be considered a negative player. However, the knockdown approach does not distinguish between the terminator and effector functions of Arf GAP because most Arf GAP proteins harbor various distinct domain(s) in addition to the Arf GAP domain. Therefore, the effects of a null mutation reflect not only the loss of Arf GAP activity but also of the protein-protein interactions determined by the other domains. In this review, we classify Arf GAP proteins as either positive or negative players in...
membrane trafficking, as well as putative terminators or effectors (see the columns labeled “roles in trafficking” and “findings related to the putative terminator functions and effector functions” in Table 1).

The most in-depth analysis of Arf GAPs as putative terminators/effectors has been carried out in ACAP1, which is involved in the Arf6-dependent formation of clathrin-coated vesicles from recycling endosomes. As cargo, the transferrin receptor is constitutively recycled back to the plasma membrane, whereas the recycling of the cell adhesion molecule integrin β1 is growth factor-dependent and that of a glucose transporter, Glut4, is insulin-dependent. In each case, ACAP1 directly binds to the above mentioned cargo molecules. Furthermore, ACAP1 can bind directly to the clathrin heavy chain and thus probably functions as an adaptor molecule by bridging the interaction between the cargo protein and clathrin. For example, the overexpression of ACAP1 inhibits the recycling of the transferrin receptor and integrin β1. This ACAP1 effect is likely due to the locking of clathrin coat protein onto membranes. Namely, an excessive amount of transduced ACAP1 appears to tether clathrin to the membrane and prevents its incorporation into vesicles. These features suggest that endogenous ACAP1 functions as a putative effector. In fact, siRNA knockdown of endogenous ACAP1 decreases recycling efficiency. siRNA-treated cells were transfected with wild-type and mutant ACAP1 and the rescue of the recycling function was evaluated. Dominant-negative and dominant-active forms of ACAP1 harbor mutations in the motif responsible for the interaction with cargo proteins, resulting in the abolishment and enhancement of the interaction, respectively. A dominant-negative form cannot rescue cargo recycling, whereas a dominant-active form can. These observations support the hypothesis that ACAP1 functions as a putative effector in the formation of clathrin-coated vesicles. However, the introduction of a GAP-negative mutant of ACAP1 into siRNA-treated cells does not rescue recycling either. Thus, ACAP1 appears to possess characteristics of both effector and terminator. In addition, the above-mentioned impairment in recycling efficiency caused by ACAP1 knockdown supports the positive role of endogenous ACAP1 in trafficking and is considered to be an overall reflection of its dual activities as effector and terminator.

The SMAP subfamily of proteins is the first Arf GAP subtype in which the binding affinity to clathrin heavy chain (and to clathrin assembly protein) has ever been demonstrated. We reported previously that the overexpression of SMAP1 inhibits the Arf6- and clathrin-dependent endocytosis of the transferrin receptor and E-cadherin. This impairment in endocytosis occurs because the AP-2 adaptor molecule is not recruited to coated pits. The overexpression of wild-type SMAP1 may induce the conversion of Arf6-GTP to an inactive Arf6-GDP form, which would inhibit the recruitment of AP-2 to the membrane. Such an interpretation is plausible as the recruitment of AP-2 to coated pits is enhanced in cells overexpressing a GAP-negative mutant of SMAP1. The above observations support a putative terminator function for SMAP1 in the sense that SMAP1 affects the recruitment of AP-2 in a GAP-sensitive way. Interestingly, the overexpression of GAP-negative SMAP1 also inhibits endocytosis of the transferrin receptor. In these cells, the recruitment of AP-2 to coated pits occurs as stated above, but that of clathrin is impaired, which could be attributed to the fact that the GAP-negative mutant still possesses an intact clathrin-binding motif. In fact, the overexpression of a SMAP1 mutant that is incapable of binding to clathrin does not inhibit the endocytosis of the transferrin receptor. These results support the putative effector function of SMAP1, which is based on its binding ability to clathrin. Thus, as in the case of ACAP1, SMAP1 likely possesses both effector and terminator functions, based on the results of overexpression experiments. However, because information on the effects of SMAP1 knockdown is incomplete, whether endogenous SMAP1 exerts a positive or negative role in the endocytosis of transferrin and E-cadherin is not clear.

In the research on the role of SMAP2, overexpression and knockdown experiments have yielded opposite results. SMAP2 depletion enhances the anterograde transport of vesicular stomatitis virus (VSV)-G protein from the trans-Golgi network (TGN) to the plasma membrane, whereas SMAP2 overexpression decreases the transport efficiency. Endogenous SMAP2 can therefore be considered a “negative” player in the TGN-to-plasma membrane pathway. Whether SMAP2 functions directly as an antagonist in the formation of clathrin-coated vesicles or if the intracellular depletion of SMAP2 leads to vesicle formation compensated by a second, strongly positive-functioning Arf GAP subtype is not clear to date.

Overexpression of the wild-type form of another Arf GAP protein, GIT1, but not its GAP-negative form, inhibits recycling of the thyrotropin receptor, suggesting a putative terminator function for GIT1. However, in both GIT1 and GIT2, which are important Arf GAPs involved in the agonist-dependent endocytosis of G-protein-coupled receptors, only one type of overexpression experiments have been reported and information on siRNA-mediated knockdown is not available. Therefore, the role of these proteins as positive or negative regulators of endocytosis is difficult to determine.

On the other hand, knockdown experiments revealed that AGAP1 and AGAP2 play positive roles in the recycling of the muscarinic receptor and the early endosome-to-TGN transport of Shiga toxin, respectively. The direct interaction of AGAP1 with the muscarinic receptor is necessary for its efficient recycling, suggesting that AGAP1 functions as a putative effector. In parallel, the overexpression of AGAP1 and AGAP2 results in the dissociation of the adaptor molecules AP-3 and AP-1, respectively, from the membrane, which disrupts the AP-3-dependent transport of LAMP1 to the lysosomes and the AP-1-dependent recycling of the transferrin receptor. These dissociation phenomena are GAP activity-dependent, suggesting a putative terminator function for AGAPs.

The possible involvement of ARAP1 in the transport of epidermal growth factor receptor (EGFR) was examined using siRNA-mediated knockdown. In one report, ARAP1 knockdown accelerated the endocytosis of EGFR from the plasma membrane, suggesting a negative role of endogenous ARAP1 in endocytosis, whereas in another report, knockdown led to the accumulation
### Table 1. Positive or negative roles exerted by various Arf GAPs in vesicle transport

| Arf GAP | Roles in trafficking | Arfs involved | coat protein | cargo | transport pathways | Types of experiment | Findings related to the putative terminator functions and effector corepressor | References |
|---------|---------------------|---------------|-------------|-------|--------------------|---------------------|------------------------------------------------------------------|------------|
| ACAP1   | positive Arfβ       | clathrin      | integrin β1 | Glu4  | constitutive recycling from endosomes to PM | KD by siRNA          | ADAP1 directly binds to transferrin receptor and promotes its transport from recycling endosomes. | 5          |
|         |                     |               |             |       |                    |                     | Disrupting activities of either ADAP1 or Akt signaling, or their assembly with endosomal integrin β1 inhibits recycling of integrin β1. Akt signaling induces phosphorylation of ACAP1, and phosphorylated ACAP1 is now capable of associating with integrin β1 directly. KD of endogenous ACAP1 and overexpression of dominant negative ACAP1 (S55A) inhibits recycling, whereas overexpression of dominant active ACAP1 (S55E) accelerates recycling. | 6          |
| SMAP1   | negative Arf1       | SV3-G         | transport from TGN to PM | KD by gene targeting and overexpression | SMAP2-depletion enhances the VSV-G transport from TGN to PM, whereas SMAP2-overexpression decreases it. | 11          |
|         |                     |               |             |       |                    |                     | Overexpression of SMAP1 inhibits endocytosis of transferrin receptor and E-cadherin. | 8, 10       |
| QT1     | negative Arfβ       | EGFR, EGF R   | endocytosis |       |                    |                     | Overexpression of QT1 reduces agonist-dependent internalization of several GPCR and EGFR (but not transferrin receptor). | 13, 15      |
| AGAP1   | negative Arfβ       | EGFR          | transport from late endosomes to lysosomes | Overexpression | Overexpression of AGAP1 impairs normal LAMPI trafficking, by directly interacting with and dissociating AP-3 from endosomal membranes. AP-3 dissociation is GAP activity-dependent. | 18          |
| AGAP2   | positive Arf1       | Shiga toxin, cholera toxin, TGN46, NBS1 | transport from early endosomes to TGN | KD by siRNA | depletion of AGAP2 blocks the retrograde transport of Shiga toxin from early endosomes to TGN. Note that overexpression of an activated form of Arf1 and that of wild type AGAP2 similarly cause a block of retrograde transport. | 17          |
| AGAP3   | positive Arfβ       | EGFR          | recycling from endosomes to PM | overexpression | Overexpression of AGAP2 re-distributes AP-1 from TGN to recycling endosomes in a GAP-dependent manner, and accelerates Raf-1-dependent exit of vesicle transport from the recycling compartment. AGAP2 associates with AP-1 directly. | 19          |
| ARAP1#  | negative Arfβ       | EGFR          | EGFR-dependent endocytosis of EGFR from PM to Rab5-early endosomes | KD by siRNA | Decreasing ARAP1 levels accelerates endocytosis of EGFR (and so a result, increases both association of EGFR with EEA1+ early endosomes and degradation of the EGFR-EEA1 complex). | 20          |
| ARAP2#  | positive Arfβ       | EGFR          | sorting endosomes to late endosomes/MVB/lyosomes | KD by siRNA and overexpression | ARAP1 KD impairs the trafficking of EGFR from sorting endosomes to a degradation pathway, resulting in the accumulation and delayed degradation of EGFR. In contrast, overexpression of ARAP1 accelerates degradation of EGFR. | 21          |
| ASAP1   | negative Arfβ       | EGFR          | recycling from endosomes to PM | overexpression | Overexpression of ASAP1 accelerates Rab11-dependent EGFR recycling. | 22, 23       |
| ASAP2   | positive Arfβ       | CD55          | endocytosis | KD by siRNA and overexpression | ASAP2 silencing as well as its overexpression reduces the internalization of CD55. | 24          |
| ASAP3   | positive Arfβ       | caveolin      | endocytosis | overexpression | Inhibition of both is enhanced in ASAP2-deficient cells. | 10          |
| ADFG1   | positive Arfβ       | transferrin receptor, VAMP7 | endocytosis | KD by siRNA | Inhibition of the expression of ADFG1 partially impairs transferrin receptor and VAMP7 endocytosis. | 26          |
| ArfGAP1 | positive Arfβ       | transferrin receptor, CPTTR | endocytosis | KD by siRNA and overexpression | ArfGAP1# directly interacts with AP-2 and transferrin receptor. KD of ArfGAP1 by siRNA reduces Arfβ- and AP-2-dependent endocytosis of transferrin receptor. This reduction can be rescued by the wild-type ArfGAP1 but not by the mutant incapable of interacting with receptor. The GAP-negative mutant cannot rescue endocytosis either. | 25          |

*When knockdown experiments of respective Arf GAP results in a decrease/abolishment of trafficking involved, then that Arf GAP is assigned to play a positive role. If the opposite result is obtained, the Arf GAP is assigned as a negative player. Note that these classifications are just tentative. **See the text as for the definition of putative terminator and effector. ***ARAP1-KD is reported to accelerate and delay the EGF-R degradation in references 20 and 21, respectively. These controversial observations remain to be clarified, since the employed experimental conditions appear similar (the same HeLa cells, siRNA purchased from the same company, the same incubation period with siRNA, and similar ranges of serum starvation period, EGF concentration and incubation period with EGF). The only difference might be the use or not of cycloheximide, although it is not known if this agent might explain the difference of results. bsa, bovine serum albumin; CFT, cystic fibrosis transmembrane conductance regulator; EGF, epidermal growth factor receptor; GPCR, G protein-coupled receptor; KD, knockdown; M6PR, mannose 6-phosphate receptor; MVB, multivesicular body; PM, plasma membrane; VSV, vesicular stomatitis virus.
of endocytosed EGFR in sorting endosomes, suggesting a positive function in the sorting endosome-to-late endosome pathway. Thus, the same ARAP1 appears to exert either a negative or a positive function depending on the trafficking pathway under consideration (see the footnote to Table 1 concerning EGFR degradation).

The final Arf GAP subtype to be mentioned is the ASAP family of proteins. Overexpression of ASAP1 enhances EGFR recycling, while the overexpression of a GAP-negative mutant of ASAP1 or that of an ASAP1 mutant incapable of interacting with its effector molecule, CIN85, fails to enhance recycling.22 CIN85 is an adaptor protein that interacts with Cbl to mediate EGFR downregulation. ASAP1 also contains a BAR-domain and, through its binding to the lipid bilayer, it can induce the formation of tubular structures. The formation of a stable complex between Arf1-GTP and the ASAP1-Arf GAP domain (but not GAP activity itself) is necessary for this tubule formation.23 ASAP2 binds to Arf6-GTP (but does not exhibit GAP activity, similar to ASAP1) and to amphiphysin I1m, contributing to the formation of a ternary complex.24 By contrast, the overexpression of ASAP3 simultaneously inhibits CD25 endocytosis25 and enhances the internalization of the bovine serum albumin receptor.18 These studies suggest that the putative terminator functions and effector functions might be attributed to ASAP family proteins.

In summary, among the various Arf GAP family members involved in the formation of clathrin-coated vesicles, ACAP1 is the most extensively analyzed subtype with respect to its putative terminator/effector functions. Although endogenous ACAP1 functions to enhance the efficiency of recycling through endosomes, the overall activity of ACAP1 as a positive element is likely to be the result of a combination of its terminator and effector functions.5-7 In the other Arf GAP subtypes, overexpression and/or knockdown experiments have suggested their possible contributions as putative terminators and/or effectors. A recent discovery of ArfGAP1 involvement in clathrin-vesicle formation25 is particularly noteworthy because it suggests a possible analogy between ArfGAP1 and Arf GAP members. ArfGAP1, an essential component in COPI-vesicle formation, is now admitted to directly interact with AP-2 as well with the transferrin receptor. Knockdown of ArfGAP1 by siRNA reduces AP-2-dependent and coated pit-mediated endocytosis of the transferrin receptor, which can be rescued by wild-type ArfGAP1 but not by an ArfGAP1 mutant that cannot interact with the receptor. The GAP-negative mutant cannot rescue endocytosis either. Thus, the findings reviewed in this mini-blog provide a basis for the elucidation of the mechanisms underlying the regulation of the terminator/effector functions of Arf GAPs using clathrin-engaged proteins as analytical tools.

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Note

Throughout the text, the term Arf GAP (a space inserted between Arf and GAP) refers to a gene family or protein domain, whereas Arf GAP (no space between Arf and GAP) refers to ArfGAP1-3 only.

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