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Review

Arf GTPase regulation through cascade mechanisms and positive feedback loops

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ABSTRACT

Arf GTPases, together with Rab GTPases, are key regulators of intracellular membrane traffic. Their specific membrane targeting and activation are tightly regulated in time and space by guanine nucleotide exchange factors (GEFs). GEFs are multidomain proteins, which are under tight regulation to ensure fully coordinated and accurate membrane traffic events. Recently, two Arf GEFs, Sec7 and Arno, have been shown to be part of Arf GEF cascades similar to the Rab GEF cascades. Both GEFs are autoinhibited in solution and require an active Arf molecule to be recruited to the membrane and to switch to an open conformation. As such, positive feedback loops, whereby the amount of Arf-GTP on a given organelle increases not linearly with time, can be established.

1. Introduction

Every cell is separated from the external medium by a plasma membrane, which acts as a protective barrier and allows controlled exchanges essential for survival. In eukaryotic cells, additional compartments are present including the endoplasmic reticulum (ER), the Golgi, endosomes and lysosomes. Each organelle is separated from the rest of the cell by a bilayer membrane, which thus defines a micro-environment with specific functions and contents. However, membrane-bound organelles are not independent: membrane and secreted proteins as well as lipids are synthesized in the ER and/or in the Golgi apparatus and then need to reach their target organelle. Protein and lipid transport is supported by a myriad of vesicles, which travel bidirectionally and rapidly from one organelle to the other, a process referred to as intracellular trafficking.

Vesicular traffic can be divided into four major steps: the budding of the vesicle from the donor membrane, its transport, its tethering and then its fusion with the acceptor membrane. Specific protein machineries including various coat proteins, motor proteins, tethering factors and fusogenic Snare proteins control these steps. Many of these proteins are peripheral membrane proteins and are directly recruited from the cytosol to the surface of their target compartment in a highly regulated spatial and temporal manner. Two major key regulators of these recruitment processes are specific phosphoinositides [1] and the small GTPases of the Arf and Rab families [2].

Arf and Rab GTPases are molecular switches, which toggle from an inactive cytosolic form (bound to GDP) to an active membrane-associated form (bound to GTP). This feature enables them to recruit effectors only when associated with a specific membrane. Thus Arfs and Rabs have a central role in determining when and where effectors are recruited to organelles. The GDP/GTP switch is intrinsically very slow and requires catalysis by proteins called guanine nucleotide exchange factors (GEFs). Because GEFs are responsible for the activation of GTPases, they participate in determining their localization. Understanding how GEFs are regulated is therefore of central importance to understand how GTPases achieve specificity and directionality in intracellular membrane traffic [3].

A well-described regulatory mechanism for Rab GTPases has been termed Rab GEF cascade. This mechanism was first described in the yeast secretory pathway by the Novick lab [4], and seems to be a general principle conserved during evolution since similar cascades have been also documented in mammalian cells [5–7]. Furthermore, the same regulatory mechanism has been recently reported for Arf GTPases [8–13]. This review discusses the two Arf GEF cascades reported to date, involving the cytohesin GEF

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Arno and the Golgi yeast GEF Sec7, and compares them to the Rab GEF cascades.

2. Arf GEF cascades

The ADP-ribosylation factor (Arf) family, including the Arf-like proteins (Arl), regulates membrane traffic by ensuring important functions including the recruitment of coat proteins to allow vesicle budding, the recruitment and activation of lipid-modifying enzymes to modify the membrane lipid composition and the interaction with actin cytoskeleton factors [14]. There are three Arf isoforms in the yeast *Saccharomyces cerevisiae* and six in mammalian cells. Based on their sequence homology, they can be divided into three classes. Classes I (Arf1, 2 and 3) and II (Arf4 and 5) play their role essentially at the Golgi whereas Arf6, the only member of the class III, is at the plasma membrane. Arf proteins are characterized by the presence of an amino-terminal amphipathic helix carrying a myristate anchor [15,16]. This helix is buried in a hydrophobic pocket in the GDP conformation. Replacement of GDP by GTP promotes the exposure of the helix thereby allowing the protein to interact sustainably with lipid membranes in the GTP conformation. This replacement also enables the reorganization of the classic "switch domains", creating an interface for the binding of effectors. As such, activation of Arf proteins and membrane recruitment are directly coupled.

Arf GEFs promote Arf activation *via* their conserved catalytic Sec7 domain, first identified in the budding yeast protein Sec7 [17]. Since Arf GEFs are essential for the localization of active Arf proteins, their own membrane targeting needs to be tightly regulated. The mechanisms that control GEFs recruitment are diverse, complex, combinatory and involve the domains that flank the Sec7 domain. Overall this allows exquisite tuning of Arf activation.

Recently, different studies have highlighted that two Arf GEFs, Arno and Sec7, can mediate cascades, which are quite similar since they involve a switch from a closed to an open conformation during GEF membrane recruitment and are regulated by a positive feedback loop.

2.1. Membrane recruitment under control of activated Arf proteins

The GEF Arno belongs to the cytohesin family, which is only present in metazoans and represents the simplest Arf GEF family. Arno contains an N-terminal coiled-coil region (CC), a central Sec7 domain and a C-terminal PH (pleckstrin homology) domain (Fig. 1). Numerous studies showed that the PH domain is responsible for the interaction of Arno with lipids and governs its cellular localization. The PH domain contains a basic pocket for phosphoinositides and splice variants are either specific for phosphatidylinositol-3,4,5-triphosphate (PIP3) or bind equally well to phosphatidylinositol-4,5-biphosphate (PIP2) [18]. In addition, a polybasic tail downstream of the PH domain interacts with negatively charged lipids such as phosphatidylserine (PS) [19]. Thus, the PH domain of Arno binds to a combination of lipids (PS + PIP2/PIP3) present at the plasma membrane. In many cell lines, however, full-length Arno is mostly found in the cytosol, although some enrichment at the plasma membrane is observed [20]. This cellular localization leads also to a dilemma because the Sec7 domain, which is responsible for the exchange activity, is, *in vitro*, much more active on Arf1, which is localized predominantly at the Golgi, than on Arf6, which is found at the cell periphery [21,22]. The biochemical properties of the Sec7 domain and of the PH domain of Arno are somehow conflicting when considered in a cellular context.

In 2007, three different publications suggested for the first time that some Arfs in their active GTP conformation, namely Arf6-GTP and Arl4-GTP, are able to recruit Arno to the membrane by binding directly to its PH domain [8,12,11]. Cohen et al. showed that the PH domain of Arno is both necessary and sufficient to recruit Arno to the plasma membrane in HeLa cells and could co-immunoprecipitate this PH domain with Arf6 or an active Arf6 mutant but neither with an inactive Arf6 mutant or Arf1 [8]. With a similar approach, Hofmann et al. showed that the PH domain of Arno is also able to bind specifically to the activated form of Arl4 [12]. So the PH domain of Arno is not only able to interact with phosphoinositides but also with activated forms of Arf GTPases. Although a PH–Arf interaction was previously described (for the PH domain of FAPP [23]), the novelty here is that this interaction occurs in the context of an Arf GEF. Traditionally, Arf GEFs are believed to control the membrane association of Arf GTPases; these new studies suggest a reciprocal effect where Arf proteins are themselves able to recruit GEFs to the membrane.

Additionally, Cohen et al. showed in Hela cells that Arno clearly prefers Arf1 as a substrate rather than Arf6 and that the coexpression of Arf6 and Arno leads to Arf1 recruitment to the plasma membrane [8]. Together, these results suggest an Arf6 > Arno > Arf1 cascade and reconcile the dilemma associated with the preferred localization of Arno and its specificity for some Arf subtypes.

The GEF Sec7 is the single member of the BIG family of Arf GEFs in yeast and is responsible for the activation of Arf1 and Arf2 at the *trans*-Golgi network (TGN). This GEF has two orthologs in mammalian, BIG1 and BIG2, and is really "big" with a molecular weight of ≈200 kDa compared to 45 kDa for Arno. In fact, the homology with Arno is restricted to the central catalytic Sec7 domain. From N- to C-terminus, Sec7 has a DCB domain (dimerization and cyclophillin binding), a HUS domain (homology upstream of Sec7 domain), a Sec7 domain and, finally, 4 HDS (homology downstream of Sec7) domains (Fig. 1) [24]. None of these domains, except the Sec7 domain, is characterized and displays an obvious membrane-binding region. So little is known about Sec7 membrane targeting, and the difficulty in purifying full-length Sec7 makes this question even harder to address. Recently, Richardson et al. purified a truncated but fully functional construct of Sec7. They show that lipids are not sufficient for membrane recruitment of Sec7 in contrast to Arno [13,25]. In addition, they present *in vitro* and *in vivo* evidence that the presence of activated Arf1 is a prerequisite for Sec7 membrane targeting *via* interaction with its HDS1 domain and that this domain is able to mediate Sec7 localization to the TGN. Other lines of evidence suggest that the full TGN localization of Sec7 is not only due to this HDS1–Arf1 interaction but involves other protein interactions. Two suggested binding partners for Sec7 orthologs are the ubiquitin ligase Rsp5 and the small GTPase Arl1 [26,27].

The aforementioned studies of Arno and Sec7 highlight that Arf GEFs are not only exchange factors for Arf proteins but also Arf effectors and thus combine two binding sites for Arf proteins, one regulatory and one catalytic (Fig. 2A).

2.2. Membrane translocation induces autoinhibition relief

In 2007, the Lambright lab discovered another important feature of the cytohesin GEF family: that these proteins adopt an autoinhibited conformation in solution. First, the crystal structure of the Sec7-PH tandem of Grp1 reveals that the linker between the Sec7 and the PH domains together with the C-terminal helix block the Arf binding site of the Sec7 domain [9]. Second, for all cytohesin members (including Arno), removal of the C-terminal region increases the exchange activity in solution up to 30-fold.

How is the autoinhibition of cytohesin GEFs relieved? To address this question in a relevant environment, i.e., a membrane surface, we recently performed reconstitution experiments using

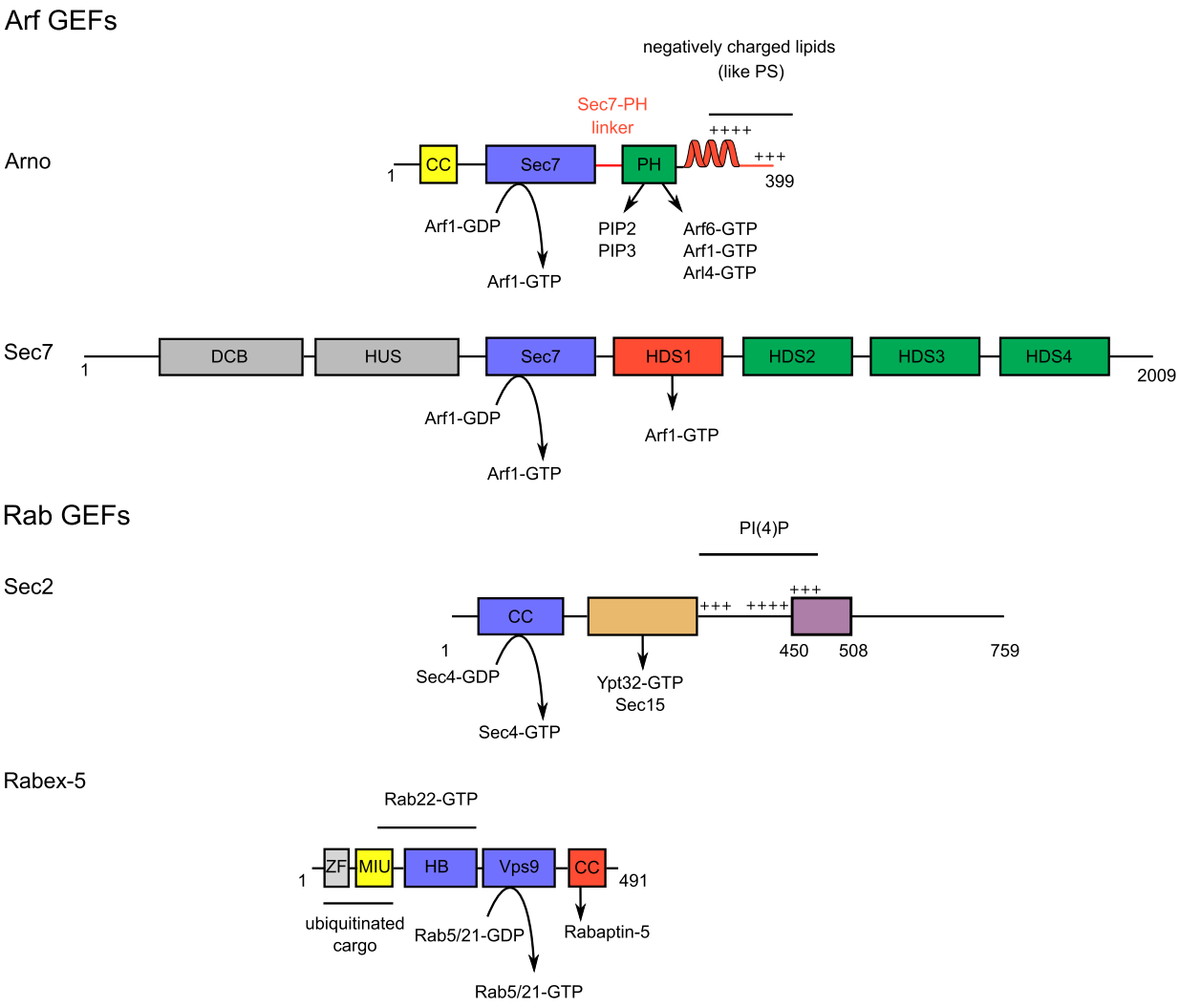

Fig. 1. Domain organization of the Arf guanine nucleotide exchange factors (GEFs) Arno and Sec7 and the Rab GEFs Sec2 and Rabex-5. The catalytic domain is indicated in blue. This domain is surrounded by different regulatory domains. The region responsible for the autoinhibition of the GEF activity is shown in red. Abbreviations are: CC, coiled-coil; PH, pleckstrin homology; DCB, dimerization and cyclophillin binding; HUS, homology upstream of Sec7; HDS, homology downstream of Sec7; ZF, zinc-finger; MIU, motif interacting with ubiquitin; HB, helical bundle.

full length Arno and as myristoylated Arf proteins on liposomes of defined composition [10]. In such systems, Arf6-GTP acts in strong synergy with lipids to activate Arno: depending on the proteins and lipids present on the liposomes, the exchange activity of Arno on Arf1 varies 500-fold. Additionally, Arf6-GTP is a much more potent activatorof Arno on lipid membranes than in solution (300-fold increase in efficiency), showing the importance of a membrane interface for proper conformational changes.

The crystal structure of full-length Sec7 is not known but *in vitro* data strongly suggest that, like Arno, Sec7 switches from an autoinhibited form in solution to a highly active form when bound to a membrane [13]. This switch is under control of the HDS1 domain, which might alternatively sequester the GEF domain in solution or release it in presence of an activated Arf protein bound to a membrane.

Several examples of autoinhibition for different GEFs families are well documented and it seems more and more likely that this regulatory mechanism is general. For example, Epac2, a GEF for Rap1B, which is important for cell adhesion and insulin secretion, is directly regulated by the second messenger cAMP. This protein has an N-terminal regulatory region, containing two CNB (cyclic-Nucleotide-Binding Domain A and B) domains, and a C-terminal catalytic domain consisting of a Cdc25-homology domain, which is stabilized by a REM (Ras Exchanger Motif) domain. The structure of Epac2 was solved in the presence or the absence of cAMP. In absence of cAMP, the CNB-B domain prevents access to the Cdc25-homology domain. Upon cAMP-binding, the CNB-B domain swings, allowing Rap1B to approach the catalytic site [28,29]. Another well documented example of autoinhibition is Vav, a Rho GEF for Rac, which plays an important role in actin regulation. Vav possesses a catalytic DH domain surrounded by several regulatory domains. NMR data show that a helix from an N-terminal extension blocks the catalytic site and that phosphorylation of a tyrosine promotes helix unfolding, hence facilitating access of the substrate [30]. An additional regulatory level is exerted by a CH domain (calponin homology), which strengthens Vav autoinhibition [31]. The relief of Vav autoinhibition occurs gradually *via* sequential phosphorylations.

2.3. Positive feedback loops

So far we have considered mechanisms whereby two different Arf proteins (e.g., Arf6 and Arf1) act, respectively, as activator and substrate of a GEF. However, the study of an Arno mutant

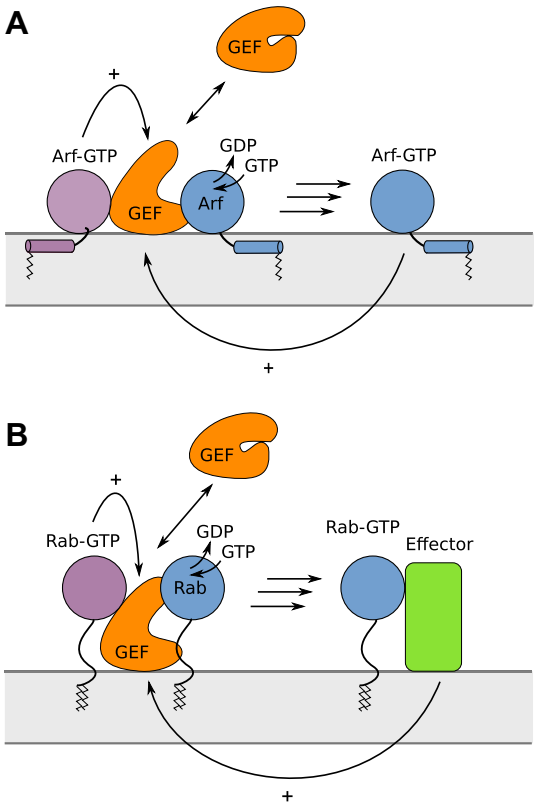

Fig. 2. Model for Arf and Rab GEFs cascades and their regulation by positive feedback loops. (A) Arf GEF cascade. An Arf GEF is recruited and switch from a close to an open conformation at the lipid bilayer thanks to an activated, GTP bound, Arf molecule. After nucleotide exchange on the substrate Arf, the newly activated Arf molecule is able to further stimulate the Arf GEF activity, thus creating a positive feedback loop. (B) Rab GEF cascade. The Rab GEF is recruited to the membrane by an activated Rab protein. Some lines of evidence indicate that, like for Arf GEFs, this membrane recruitment is accompanied by a conformational change. The activation of the downstream Rab by the Rab GEF leads to the recruitment of several effectors. One of these effectors binds in turn the Rab GEF and further stimulates its exchange activity.

led to the discovery of an alternative mechanism, whereby these two functions are fulfilled by the same Arf subtype [10]. The Arno K336A mutant is not able to interact with Arf6-GTP because of a point mutation in the PH domain. The K336A mutation is puzzling because it totally prevents Arno from activating Arf1 on a membrane surface despite the facts that it affects neither the interaction of Arno with lipids nor its level of autoinhibition. Because the kinetics of Arf1 activation by Arno in the absence of Arf6-GTP displayed a sigmoidal shape, we foresaw that Arf1-GTP could also activate Arno by interacting, like Arf6-GTP, with its PH domain. To test this positive feedback loop hypothesis, we used three different strategies: the addition of increasing amounts of Arf1-GTP in assays where the activation of Arf1-GDP by Arno was followed; the use of mant-derivatives of GDP and GTP to check if futile GDP to GDP exchange on Arf1 by Arno is less favorable than GDP to GTP exchange; and, lastly, the use of Arf effectors which, by binding to newly formed Arf1-GTP molecules, should prevent Arf1-GTP from stimulating Arno. These three different approaches lead to the same conclusion: Arno is activated by Arf1-GTP, albeit less efficiently than by Arf6-GTP. Altogether, these *in vitro* reconstitutions suggest that the presence of an activated Arf molecule is mandatory for the activation of Arno and that this role can be fulfilled by not only Arf6-GTP but also Arf1-GTP (Fig. 2A). Further structural work will be required to determine the exact mechanism by which Arno opens at the membrane surface *via* interactions between its PH domain and ArfGTP.

Since the membrane recruitment of Sec7 is mediated by Arf1-GTP, which is also the product of Sec7 activity, Richardson et al. addressed the possibility of a positive feedback loop in the case of Sec7 [13]. With a similar *in vitro* approach as for Arno, they show that adding increasing amounts of Arf1-GTP stimulates the exchange activity of Sec7 on Arf1 at the surface of artificial membranes. Furthermore, the kinetics of Arf1 activation by Sec7 displays a lag phase before maximal activity. These results demonstrate a positive feedback between Arf1 and Sec7, whereby Sec7 activates Arf1, which in turn activates Sec7.

To date, the only other (and actually first) example of a GEF regulated by a positive feedback loop is the Ras exchange factor Sos. During a crystallographic investigation, the laboratories of Kuriyan and Bar-Sagi made the surprising discovery that Sos has a second binding site for Ras that is specific for the GTP form and distal from the catalytic site [32]. Like for Arno and Sec7, Ras-GTP, the product of the reaction, binds to a regulatory domain of Sos and stimulates its activity. However, the catalytic domain, which is a Cdc25 domain, is not obstructed like the Sec7 domain of Arno, and the binding to Ras-GTP does not promote relief of autoinhibition but rather stabilizes Ras in the catalytic site of Sos, resulting in better activity.

2.4. In vivo significance

The cytohesin GEF Arno, which only exists in metazoans, does not seem to be involved in constitutive functions, such as controlling the formation of transport vesicles, but rather in processes that require large membrane rearrangements like dendritogenesis [33], cell migration [34], phagocytosis [35], macropinocytosis [8] and organism growth controlled by insulin [36,37]. Recently, a study revealed also a key role of Arno during *Salmonella* invasion [38]. It is tempting to make a link between these functions and the biochemical properties of Arno, i.e., its tight autoinhibition, the dependence of its activation on the presence of Arf-GTP and its regulation by a positive feedback loop. In resting cells, Arno probably remains inactive because the level of Arf-GTP molecules is limited. Indeed, by interacting with classical effectors, Arf-GTP molecules, which ensure elementary functions like vesicular transport at the Golgi in the case of Arf1 or endocytosis at the plasma membrane in the case of Arf6, may be present in very limiting amounts. As such, Arno would remain locked in a closed conformation. An experiment in RPE1 cells shows that an Arno mutant that is less dependent on the presence of free Arf-GTP disturbs the subcellular localization of Arf1, leading to uncontrolled activation of Arf1 in cells [10]. This experiment suggests that tight autoinhibition of Arno is key for preventing it from activating Arf1 without the guidance of Arf6/Arl4. Interestingly, high expression levels of Arno are found in primary human lung adenocarcinomas [39]. Additionally, administration of SecinH3, a cytohesin inhibitor, to nude mice bearing lung cancer xenographs reduces tumor growth.

A large burst of Arf6-GTP (or Arl4-GTP) at the plasma membrane acts probably as a trigger for Arno activation. What triggers this burst is unknown but studies on insulin signaling have identified interesting protein partners of Arno and Arf6 [37,40]. Since the presence of PIP2/PIP3 and PS is also essential for Arno recruitment, Arno can only fulfill its function at the plasma membrane. Once activated, Arno activates in turn Arf1 at the plasma membrane and, through the positive feedback, maintains its own activity by substituting Arf1-GTP for Arf6-GTP (or Arl4-GTP), the former being much more abundant in the cell than the latter. Positive feedback loops are frequently observed when cells change their behavior because they allow reactions to move forward decisively [41,42]. This fits well with Arno functions, which are associated with unusual large rearrangements of the plasma membrane. Thus, insulin triggers the relocalization of Arf1 from the Golgi to the plasma membrane in cells overexpressing Arno and Arf1 [43].

Sec7 was first identified in a screen for secretion-defective mutants in *S. cerevisiae*[44]. Temperature sensitive Sec7 mutants accumulate exaggerated TGN membranes. This observation fits with a function of Sec7 at the TGN in promoting anterograde traffic out of this compartment. Richardson et al. highlight that Sec7 is initially recruited to the TGN by direct interaction with Arf1-GTP [13]. It remains to be determined which binding partner(s) achieve(s) this specific localization of Sec7 at the TGN since Arf1 is present throughout the Golgi and since membrane recruitment of GEFs is generally achieved through multiple interactions. Alternatively, the interaction of Arf1-GTP with Sec7 might be incompatible with the interaction of Arf1-GTP with the COP1 coat, which occurs at the early Golgi. Consequently, Arf1 might interact with Sec7 only at the TGN [45]. Once recruited, Sec7 activates Arf1, which establishes a positive feedback loop to further stimulate Sec7 activity. In this case, regulation by a positive feedback loop is linked to a constitutive function: building up highly concentrated Arf1-GTP domains in order to recruit many effectors at the TGN to generate different vesicles and transport intermediates.

3. Similarities with Rab GEF cascades

To date, 11 Rabs have been identified in yeast and over 60 in mammalian cells, reflecting the complexity of intracellular traffic pathways in higher eukaryotes. Rab proteins are implicated in most major steps in membrane traffic, from the budding of vesicles from donor membranes, to the fusion of vesicles with the acceptor membranes. When associated with their target organelle, Rabs recruit a wide range of effectors such as molecular motors and tethering factors [46]. Each organelle carries a specific set of Rab proteins, which contribute to define organelle identity. In contrast to Arf proteins, which are closely associated with the lipid bilayer, Rab proteins are believed to "float" about 7 nm over the lipid surface due to a flexible hypervariable C-terminal region. Two prenyl groups are covently attached to the C-terminus and anchor the protein to the membrane surface. In the GDP-bound state, Rabs can form a complex with GDI (GDP-dissociation inhibitor), which masks the prenyl groups, thereby keeping Rab-GDP in the cytosol. Membrane proteins, called GDI displacement factors (GDF), dissociate the Rab from the GDI, resulting in the association of the Rab with the membrane *via* its prenyl groups. At the membrane, Rab proteins switch to their active GTP form by interacting with specific GEFs. As for Arf proteins, GTP induces a conformational change in the switch regions allowing Rab proteins to interact with their effectors [47,2,48]. According to this cycle, GEFs are, as in the case of Arf proteins, key determinants for the localization of active Rab proteins. A very recent study strengthens this idea since mistargeting of Rab GEFs to mitochondria leads to a redirection of Rab proteins to the same compartment [49]. Surprisingly, effectors can also contribute to Rab specific localization. In the following section, we focus mainly on two Rab GEFs, Sec2 (and its mammalian homologue Rabin8) and Rabex-5, which drive membrane maturation *via* Rab cascade mechanisms. For a more complete review on these mechanisms, see [48].

3.1. Rab GEF cascades for membrane maturation

Rab5 is concentrated on early endosomes, whereas Rab11 resides on recycling endosomes, and Rab7 is found mostly on late endosomes. Of note, several distinct Rabs can coexist on the same organelle but generally occupy distinct membrane microdomains (reviewed in [50]). Therefore, complex combinations of Rabs contribute to organelle function and identity. Because intracellular traffic is very dynamic and because organelles undergo maturation, the challenge for the cell is to maintain a specific distribution of Rab proteins. To achieve this task, one key mechanism is the Rab GEF cascade, a mechanism that is based on the fact that an effector of an upstream Rab can act as a GEF for a downstream Rab (Fig. 2B). Several Rab cascades have been described in yeast and mammalian cells and operate at distinct stages of intracellular traffic, illustrating the conservation of this mechanism [48].

3.1.1. Sec2: from Ypt32 to Sec4

The first Rab GEF cascade was identified by the Novick lab in the yeast secretory pathway and involves the Rab GEF Sec2 and two Rabs, Ypt32 and Sec4 [4]. Sec2 is crucial for exocytosis since impairing Sec2 activity results in the accumulation of vesicles in the cytoplasm [51]. Sec2 is concentrated on secretory vesicles traveling from the Golgi to the plasma membrane at sites of polarized secretion. The N-terminal part of Sec2 contains a CC domain ensuring both homodimerization of Sec2 and interaction with the Rab protein Sec4 (Fig. 1). Most importantly, the crystal structure of the CC in complex with Sec4 shows that this domain catalyzes GDP-to-GTP exchange by imposing extensive structural rearrangements in Sec4 to reduce its affinity for the nucleotide [52,53]. As for the C-terminal part of Sec2, it is crucial for cellular localization: deletions or point mutations in a 58-amino acid domain (amino acids 450–508) cause Sec2 to lose its localization and compromise exocytosis and growth at the restrictive temperature [54]. However this region is not sufficient for anchoring Sec2 on membranes. A key observation was that another Rab, Ypt32 in the GTP-bound state, is necessary to recruit Sec2 on the membrane by binding to a region downstream of the CC domain [4]. Ypt32, which is predominantly associated with the late Golgi, regulates vesicle budding out of this compartment. So, Ypt32 is the first Rab of the cascade, which, in the activated state, recruits Sec2 to the Golgi. Then Sec2 activates the second Rab, Sec4, leading to the delivery and fusion of vesicles with the plasma membrane. Importantly, the phosphoinositide phosphatidylinositol-4-phosphate (PI(4)P) acts in parallel with Ypt32 in the initial recruitment of Sec2 to the Golgi [55]. Sec2 has no conserved lipid-binding motif but three positively charged patches important for PI(4)P binding. Therefore, as in the case of Arno, a specific phosphoinositide and an activated small GTPase act in synergy to target a GEF to a specific compartment. However, there is no evidence that membrane targeting of Sec2 is coupled to autoinhibition relief, since Ypt32–GTP is not able to stimulate the activity of Sec2 on Sec4 in solution [4]. Further investigations are needed to address this question.

A few years later, a mammalian Rab cascade, Rab11–Rabin8–Rab8, which is analogous to the yeast cascade Ypt32–Sec2–Sec4, was found to coordinate ciliogenesis [5]. Rab8 is important for primary ciliogenesis and regulates membrane traffic from endosomal compartments to the cell surface [56]. Rabin8 is a specific GEF for Rab8 [57] and also interacts directly with the GTP form of Rab11 [5]. Interestingly, Rab11–GTP is able to stimulate the GEF activity of Rabin8, suggesting that Rabin8 is regulated *via* an autoinhibitory mechanism. In agreement with this hypothesis, a Rabin8 mutant lacking a small region of six amino acids (SLYNEF) that is conserved in Sec2, has increased exchange activity on Rab8 in solution [58]. Very recently, evidence has been reported that phosphatidylserine (PS) cooperates with Rab11 for the membrane recruitment of Rabin8, suggesting some differences with the yeast cascade, where PI(4)P but not PS regulates Sec2 [59].

3.1.2. Rabex-5: from Rab22 to Rab5

Rab5 is localized on early endosomes and at the plasma membrane and promotes early endosome fusion [46]. Rabex-5 has potent GEF activity towards Rab5 (and Rab21) but weak activity on Rab22. The catalytic core of Rabex-5 consists of a helical bundle and a Vps9 domain and the crystal structure of these tandem domains has been solved (Fig. 1) [60]. Rabex-5 is able to bind directly

to the GTP form of Rab22 with a region immediately upstream of the VPS9 domain [61]. This interaction results in the targeting of Rabex-5 to Rab22-containing early endosomes, an effect that is abrogated by Rab22 knockdown in HeLa cells. Thus, Rabex-5 is an effector for Rab22 and activates in turn Rab5 on early endosomes. Whereas the recruitment of Rabex-5 depends also on ubiquitinated partners, no specific lipid involved in Rabex-5 recruitment has been reported so far [62].

3.2. Positive feedback loops via GEF-Rab-effector complexes

Rab GEFs have been shown to interact directly not only with specific activated Rab proteins, but also with some of their effectors. Furthermore, effector interaction seems to increase the exchange activity of the Rab GEF, thus leading to positive feedback loop effects (Fig. 2B). This mechanism is quite general in the field of Rab GTPases and might contribute to the abrupt formation of membrane domains containing a high density of activated Rab proteins. Note that in the case of Rab positive feedback loops, effectors act as collaborators and not as competitors as observed in the case of Arf positive feedback loops.

3.2.1. Rab5–Rabex-5–Rabaptin-5. Feedback loop

Rabaptin-5, a Rab5 effector, is essential for endosomal fusion. The recruitment of Rabaptin-5 to early endosomes is dependent on its interaction with the GEF Rabex-5 [63]. In turn, Rabaptin-5 is able to stimulate the exchange activity of Rabex-5 [64]. These observations suggested for the first time the formation of a ternary GEF–Rab–effector complex. This would establish a positive feedback loop whereby Rabex-5 is recruited to the membrane followed by Rabaptin-5 leading to further activation of Rab5. As such, a dense cluster of Rab5-GTP is created, favoring endosomal fusion. Delprato et al. identified in Rabex-5 an autoinhibitory element, downstream of the Vsp9 catalytic domain, which overlaps with the Rabaptin-5 binding site [60]. Rabex-5 autoinhibition can be partially reversed through the formation of a complex with Rabaptin-5. Indeed, the exchange activity of Rabex-5 increases with the concentration of Rabaptin-5 present whereas Rabaptin-5 has no effect on a Rabex-5 construct lacking the inhibitory element.

3.2.2. Sec2–Sec4–Sec15. Feedback loop

Once activated by its GEF Sec2 on secretory vesicles, Sec4–GTP is able to interact directly with Sec15, an exocyst component [65]. Interestingly, Sec15 is also able to interact directly with the GEF Sec2 [66]. A positive feedback loop is thus established that might contribute to create a microdomain of high Sec4–GTP density. Importantly, the Sec15 binding site in Sec2 overlaps with the Ypt32 binding site, implying that Sec15 and Ypt32 compete against each other for binding to Sec2 [66]. Additionally, the aforementioned 58-amino acid domain negatively regulates Sec15 binding to Sec2, and PI(4)P contributes to maintain Sec2 in such an autoinhibited conformation [55]. Overall, these results imply that activation of Sec4 does not simply result from the recruitment of Sec2 by Ypt32, but is delayed such that full GEF activity is achieved only after the vesicle pinches off and when PI(4)P on the vesicle is degraded. The conformational change in Sec2 facilitates the substitution of Ypt32 by Sec15. Thereafter, the Sec2-Sec4-Sec15 complex persists, leads to Sec4–GTP accumulation and promotes transport and tethering of the vesicle with the plasma membrane.

Rabin8 and Rab8, the mammalian homologues of Sec2 and Sec4, are also able to interact directly with the exocyst component Sec15 suggesting that the GEF–Rab–effector interaction is conserved in mammalian cells [58,67]. Furthermore, Rab11–GTP and Sec15 bind to a common region adjacent to the GEF domain of Rabin8, which seems to switch its binding specificity from Rab11 to Sec15 to achieve vesicle delivery during ciliary membrane formation [58]. Two recent studies add an additional level of regulation by showing that Rabin8 can be phosphorylated by NDR kinases. Rabin8 phosphorylation seems important for dendrite branching, spine development and ciliogenesis [68,59]. The phosphorylation site lies immediately downstream of the catalytic domain in the region of Rabin8 crucial for binding of Rab11–GTP and Sec15. Interestingly, a phosphomimetic mutation of Rabin8 inhibits binding to PS and promotes binding to Sec15 [59]. It seems that phosphorylation helps Rabin8 to interact with the exocyst in order to promote primary cilium formation. In yeast, phosphorylation of Sec2 has been also suggested, although the phosphorylation site(s) have not been determined and the identity of the kinase remains hypothetical although some evidence points to Cbk1 [54,69].

4. Conclusions

The discovery of cascades involving small GTPases supports the idea that membrane traffic pathways do not correspond to a collection of isolated sub-reactions, each specific to a given organelle, but rather to a large orchestration of reactions interconnected by feedback loops. In this review, we have described and compared the two Arf GEF cascades reported to date and some Rab GEF cascades. Although Arf GEFs and Rab GEFs are structurally very different, some recurrent themes emerge, including the use of two binding sites for the small G protein, one catalytic and one regulatory, the presence of autoinhibitory regions, and the possibility of additional levels of control through interactions with lipids and covalent modifications, notably phosphorylation. Dissecting the conformational changes that allow Arf and Rab GEFs to gradually change their catalytic activity over three orders of magnitude will require further structural work accompanied by complex reconstitutions on artificial membranes to decipher the contribution of each mechanism.

Feedback loops are key for making cell-signaling systems almost irreversible, an important property in the case of events such as the cell cycle [42]. Biochemical pathways, called “bistables”, are self-sustaining and provide non-linear responses. Some lines of evidence indicate that membrane maturation along the vesicle pathway follows such a bistable behavior, thanks to the combination of both positive and negative feedback loops. Using fast live-cell imaging, the Zerial lab showed that the level of Rab5 on early endosomes increases until it reaches a maximum and then suddenly drops. This decline is followed by an increase in Rab7 leading to the conversion of early to late endosomes [6]. This process is now modeled and the authors proposed that Rab5 activates Rab7 until Rab7 reaches a threshold upon which it inactivates Rab5 through a negative feedback loop [70]. Further investigation show that SAND-1 act as a critical switch in the Rab5-to-Rab7 conversion by interrupting the positive feedback loop of Rab5 through displacement of Rabex-5 and recruitment of Rab7 to endosomal membranes [71].

Other scheme of regulation have been described involving GTPase-Activating proteins (GAP). GAPs regulate the molecular switch of small GTPases by stimulating the slow intrinsic hydrolysis of GTP to GDP. Rab GAP cascades probably operate in a counter current fashion with regards to Rab GEF cascades (reviewed in [48]). The basis of these cascades is that a downstream Rab recruits the GAP that inactivates the upstream Rab.

Arf cascades have not been characterized in fine details compared to Rab cascades but a promising working model is the Golgi apparatus. The Glick lab, together with a similar study of Nakano lab, visualized cisternal maturation in yeast by simultaneously tagging the early Golgi with the GDP-mannose transporter Vrg4 (fused to GFP) and the late Golgi with the Arf GEF Sec7 (fused to DsRed) [72,73]. Cisternae exhibit green fluorescence for 2–3 min,

pass through a brief transition phase (15–20 s) where both colors overlap, and then exhibit red fluorescence for 2–3 min.

The probably bistable property of membrane conversion in vesicle transport suggests that the amount of small GTPases and of their regulators vary between well-defined limits. Note that the use of overexpressed proteins or bioprobes engineered to detect activated small GTPases should affect this equilibrium, thereby disturbing feedback loops and making the *in vivo* study of these mechanisms challenging.

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