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Review

The role of ADP-ribosylation factor and SAR1 in vesicular trafficking in plants

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Abstract

Ras-like small GTP binding proteins regulate a wide variety of intracellular signalling and vesicular trafficking pathways in eukaryotic cells including plant cells. They share a common structure that operates as a molecular switch by cycling between active GTP-bound and inactive GDP-bound conformational states. The active GTP-bound state is regulated by guanine nucleotide exchange factors (GEF), which promote the exchange of GDP for GTP. The inactive GDP-bound state is promoted by GTPase-activating proteins (GAPs) which accelerate GTP hydrolysis by orders of magnitude. Two types of small GTP-binding proteins, ADP-ribosylation factor (Arf) and secretion-associated and Ras-related (Sar), are major regulators of vesicle biogenesis in intracellular traffic and are founding members of a growing family that also includes Arf-related proteins (Arp) and Arf-like (Arl) proteins. The most widely involved small GTPase in vesicular trafficking is probably Arf1, which not only controls assembly of COPI- and AP1, AP3, and AP4/clathrin-coated vesicles but also recruits other proteins to membranes, including some that may be components of further coats. Recent molecular, structural and biochemical studies have provided a wealth of detail of the interactions between Arf and the proteins that regulate its activity as well as providing clues for the types of effector molecules which are controlled by Arf. Sar1 functions as a molecular switch to control the assembly of protein coats (COPII) that direct vesicle budding from ER. The crystallographic analysis of Sar1 reveals a number of structurally unique features that dictate its function in COPII vesicle formation. In this review, I will summarize the current knowledge of Arf and Sar regulation in vesicular trafficking in mammalian and yeast cells and will highlight recent advances in identifying the elements involved in vesicle formation in plant cells. Additionally, I will briefly discuss the similarities and dissimilarities of vesicle traffic in plant, mammalian and yeast cells.

Keywords: ARF; Sar1; p23/p24 family proteins; COPI; COPII; Vesicular trafficking; Plant

1. Introduction

Newly synthesized proteins in eukaryotic cells are transported from their site of synthesis, the endoplasmic reticulum (ER), to their correct final destination through the secretory pathway. This process is initiated by the export of properly folded and assembled secretory proteins from the ER to the *trans*-Golgi network via Golgi complex, where they are sorted and transported to their final destination [1,2]. At the same time, endocytosed proteins from plasma membrane or escaped ER proteins to the Golgi complex during normal ER to Golgi transport can travel back to the ER. Thus, anterograde and retrograde transport of the biosynthetic proteins occurs simultaneously in order to achieve a balance that retains the individual character of each participating membrane [1,3,4]. Malfunction in any of the components of the pathway leads to the breakdown of cellular organelles, cell polarity, overall cellular architecture, cell function, and ultimately cell death. Each transport step between the adjacent compartments (organelles) must be tightly controlled at least four basic levels: (1) sorting of secretory cargo from residents; (2) formation and transport of cargo carriers; (3) delivery of the cargo at the acceptor membranes; and (4) recycling of the transport machinery component to the donor membrane, which is essential for subsequent rounds of transport [5].

A combination of biochemical, molecular, genetic and morphological approaches has been taken to elucidate the transport machinery of this secretory pathway. This has resulted in a widely accepted transport model in which

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small coated vesicles (around 60 to 80 nm in diameter) act as carriers that mediate uni- or bi-directional transport between two adjacent membranes in the secretory pathway [1,6–9].

The major protein components of a number of vesicular trafficking pathways have been identified and characterized. The recent availability of subsequently completed genome sequences for several eukaryotic organisms including *Arabidopsis thaliana* creates new opportunities to address the role and function of these proteins at each defined step of vesicular trafficking [10]. Recently, RNAi and antisense technologies have been used to identify the new players in secretory pathway [11,12]. This knowledge allowed us to address specific questions such as how the different cargo proteins are selectively sorted at a specific budding-site of the donor compartment and how cargo is selectively captured in a specific type of vesicle [13–15].

At the time of vesicle budding, three specific requirements have to be fulfilled in order to produce a functional vesicle. First, different coat protein complexes are required for different vesicular transport processes, and therefore correct species of cytosolic coat proteins are attracted to the donor membrane. Second, certain specific membrane proteins need to be incorporated in transport vesicles for fusion to the target compartment. For example, v-SNAREs are required for fusion with acceptor membrane [16]. Third, the vesicles, which originate from the donor compartment, must select and capture cargo for delivery to the acceptor compartment [17]. Recent work in yeast and higher eukaryotic cells suggest that all three requirements for functional vesicle budding could be regulated in a single mechanistic step, the formation of a "priming complex" of a small GTPase (Arf or Sar1), a membrane protein, and a coat subunit [17].

Three types of transport vesicle have been functionally characterized at a molecular level and can be defined by both their membrane origin and their coat proteins [18]. Clathrin-coated vesicles are formed from both the plasma membrane and the *trans*-Golgi network and mediate vesicular trafficking within the endosomal membrane system [19,20]. Both COPI- and COPII-coated vesicles are transport intermediates of the secretory pathway [1,2,4,21]. COPII vesicles emerge from the ER and export newly synthesized secretory proteins towards the Golgi [2,21]. COPI vesicles appear to be involved in both anterograde and retrograde transport within the Golgi complex [22], as well as mediating the recycling of proteins from the Golgi to the ER [23–25].

Our understanding of the mechanics of vesicle-coat assembly is entering a new era due to the analysis of three-dimensional structures. For clathrin-coated vesicles, a sufficient number of coat protein domains have been resolved to allow the structural features of selected subunit interactions to be deduced [26]. For COPI-coated vesicles, structural and biochemical studies have provided a wealth of information regarding the interactions of coat proteins and between ARF and the proteins that regulate its activity (nucleotide exchange factors and GTPase-activating proteins (GAPs)) as well as providing clues to the types of effectors that are controlled by Arf [27–30]. The crystal structures of Arf1 and the domain of GAP, which interacts with Arf1, have been determined as well [31–35]. Another key regulatory GTPase in early secretory pathway is Sar1 and functions as a molecular switch to control the assembly of COPII-protein coats that direct vesicle budding from ER [17,21,36–39]. The crystal structure of Sar1-GDP at 1.7-Å resolution has been reported [40] and recently, molecular aspects of COPII function in ER export have been addressed by determining the structure of the Sec23/24–Sar1 complex [41]. Several interacting factors with this GTPase have been identified (Sec12p as a GEF and Sec23/24–GAP) and their role in promoting COPII vesicle coating and uncoating has been established. Genetic, biochemical, and structural analyses have demonstrated that most ER export signals bind to Sec24p [13,14] and display at least three distinct binding sites for cargo recognition [13].

Despite the recent advances in understanding the mechanism of vesicular transport in the secretory system in animal and yeast cells, our knowledge about the mechanism underlying the equivalent process in plant cells is largely fragmentary. Several plant homologue proteins of secretory pathway have been identified and it appears that plants essentially use the same protein machinery for trafficking in the secretory pathway as described for animals and yeast [42–49]. It is not surprising that conservation of the basic organization of the plant secretory system is morphologically similar to that of other eukaryotes. However, the behaviour of secretory organelles such as ER and the Golgi is dramatically different in plant cells [50–52]. In this chapter, I describe the recent progress in understanding the role of two major GTP-binding proteins, Arf and Sar1, in vesicular trafficking in yeast and mammalian cells, and compare this with plant cells.

2. ADP-ribosylation factor proteins

Arfs are highly conserved, ubiquitous, 21-kDa GTP-binding proteins implicated in the maintenance of organelle structure, formation of two types of coated vesicles in the secretory and endocytic pathway and other cellular processes [28,53]. The Arfs share >60% sequence identity and share activities as cofactors in the ADP-ribosylation of $G\alpha_s$ by bacterial toxins (cholera and *Escherichia coli* heat-labile toxin) [54], activators of phospholipase D [55,56], and phosphatidylinositol 4-phosphate 5-kinase [57]. The expression of each of the five human Arf was able to rescue the lethal double mutation of *arf1*$^-$ *arf2*$^-$ in the yeast *Saccharomyces cerevisiae* [58,59]. Arf is reported to interact with a novel protein, Arfaptin-1, in a GTP-dependent manner [60]. Arfaptin inhibits Arf in both the in vitro ADP-ribosylation assay and PLD assay [61]. Members of the Arf family lack

the carboxy-terminal isoprenylation and carboxymethylation typical of other ras-related proteins and have an additional nucleotide-sensitive region, an extension at the N terminus and a covalently attached myristate that together work as a "myristoyl switch" to coordinate activation with translocation onto a membrane [62]. This key biochemical feature allows Arf to couple the GTP–GDP conformational switch to membrane binding [63]. Arf has weak structural homology with other ras-like GTP binding proteins and has specific sequence motifs, e.g., D(V/I)GGQ instead of DTAGQ as found in the other subfamilies at the second consensus GTP-binding domain [64].

Six Arf proteins have been identified in mammals, three in yeast, 12 in *A. thaliana* [28,65,66], and several Arf-related proteins have been found in mammals, yeast and plants [66,67]. The distinction between Arfs and Arf-related proteins is based on both sequence relatedness and function [68]. The Arf-like (Arl) proteins are 40–60% identical to each other or to any Arf and are essentially devoid of the activities described for Arfs [58,69,70]. More than 10 Arls have been identified in humans, 3 in *S. cerevisiae*, and 6 in *A. thaliana* [66,68,69].

All six mammalian Arf genes have been cloned. Based on phylogenetic analysis, deduced amino acid sequences, protein size, and gene structure, Arf can be divided in three classes: class-I (Arf1, Arf2, and Arf3), class-II (Arf4 and Arf5), and class-III (Arf 6) [71]. A comparison of the class I *Arf* gene sequences indicates many similarities in their gene structure [72–74]. *ARFs* of class I gene is encoded by five exons and the open reading frame of each gene is encoded by four exons (exon 2–5) having identical intron/exon boundaries. The 5′-flanking region of each gene has a high GC content and lacks TATA and CAAT motifs. The promoters of the class I *Arfs* also contain multiple GC boxes; however, deletion analysis of the *Arf3* promoter indicates that removal of the GC boxes located in the proximal promoter region do not affect its transcriptional activity. In contrast, preliminary characterization of the class II *Arfs* indicates that their coding regions are disrupted by introns at locations distinct from the class I *Arfs* [75]. Although all classes of Arfs are structurally similar and have shown to possess similar activities, particularly in in vitro assays, the cellular roles of each Arf seem to be diverse [76,77]. Class-I Arfs are currently the best understood and have been shown to regulate the assembly of several types of vesicle coat complexes including COPI on the Golgi apparatus, clathrin-AP1 on the *trans*-Golgi network (TGN), clathrin-AP3 on endosomes and the recruitment of AP-4 to the TGN [29,78,79]. Arf1 dependent association of GGAs (Golgi-associated, γ-adaptin homologous, Arf-interacting proteins) with TGN has been also reported [80,81]. Recently Arf1 has been implicated in the orchestration of mitotic Golgi breakdown, chromosome segregation, and cytokinesis [82]. Little is known about the function of class-II Arfs but their cellular localization and function appear to be similar to class-1 Arf [83,84]. Both Arf1 and Arf5 are equally effective in promoting the recruitment of the AP-1 adaptor complex in Golgi [85] and expression of either Arf1 or Arf5 genes corrects the impairment of secretion in yeast induced by Arf deletion [59]. Arf6 functions exclusively in the endosomal-plasma membrane system and is involved in peripheral vesicle trafficking such as endocytosis and exocytosis [86,87]. It plays an important role in the regulation of actin cytoskeleton and induces the formation of F-actin-rich ruffles and promoted cell spreading [88–90]. Arf6 modulates the clathrin endocytosis in Madin–Darby canine kidney (MDCK) cells [91]. It has also been implicated in Fc-mediated phagocytosis in macrophages [92] and in insulin stimulation of adipsin secretion [93] and Glut4 translocation [94].

3. Structural organization of Arf

Like all small GTP-binding proteins of Ras superfamily, Arfs bind guanine nucleotides and adopt two different conformations: an inactive GDP-bound state and an active GTP-bound state that bind selectively to effectors [95]. The crystal structures of the two most divergent human Arfs, Arf1 and Arf6 have been determined in several forms [31,32,34,96] (see Fig. 1). Arf1 shares structural homologies with Ras core domain but presents specific characteristics. A comparison of Arf bound to GDP and to a nonhydrolyzable GTP analog revealed a unique conformational switching mechanism in which GTP binding to Arf triggers the exposure of the proteins' membrane anchor—a myristoylated amino-terminal α-helix (Fig. 1B) [34]. The crystal structure of GppNHp-bound Arf1 (nonhydrolyzable analog) establishes the closer relationship between Arf and Ras GTPases particularly in the arrangement of the switch 1 and switch 2 regions that interacts with the effectors of these proteins (Fig. 1B and C). In contrast, the switch 1 and switch 2 regions in the structure of Arf bound to GDP [31,32] do not resemble other proteins of the Ras superfamily (Fig. 1A). The structure of Arf1 in the triphosphate confirmation reveals the nature of the conformational transition from the GDP-bound form [31] and the mechanism by which the presence of the γ-phosphate triggers the exposure of the myristoylated N terminus for membrane binding [34]. The structural differences between the GDP and GTP bound state of Arf1 are localized to a contiguous region of polypeptide residues 38–83 (Fig. 1A and B). This encompasses switch 1, strands β2 and β3 and the intervening loop λ3, as well as switch 2 [34]. The molecular mechanism by which the observed transition between the GDP and GTP forms controls the exposure of the N-terminus is shown in Fig 1. The pivotal feature of the GTP-myristoyl switch is the 7 Å displacement of strands β2 and β3, since this couples the binding of GTP to the extrusion of loop λ3 from the GTPase core and this movement of loop λ3 eliminates the binding site for the N terminus, which will eventually become available for membrane interaction (Fig. 1A and

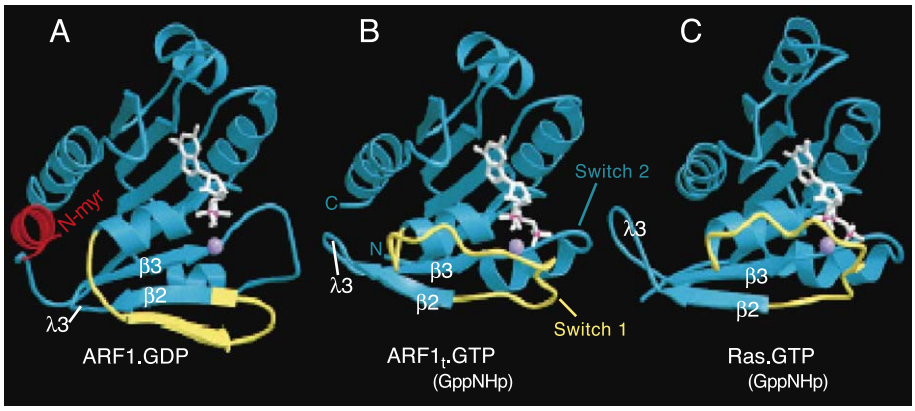

Fig. 1. Structure of Arf1 in the triphosphate conformation. A ribbon representation of Arf1 in the GDP-bound form (A) and its comparison with Arf1 in the triphosphate conformation (B) and GTP-analog-bound Ras (C). The N-terminal α-helix of GDP-bound Arf1 is coloured red. In vivo, Arf1 is posttranslationally myristoylated at its N terminus. Bound nucleotide is coloured white, with phosphorus atom pink and Mg^{+} ions drawn as magenta spheres. The switch 1 and switch 2 regions are modified during GDP–GTP exchange (please see text for details). (Reprint with permission from Goldberg [34].)

B). The elements of polypeptide sequence that participate in the structural transition are highly conserved among Arf proteins, implying that the GTP-myristoyl switch mechanism is common to all Arf-mediated membrane-dependent transactions in the cell [34].

4. Guanine nucleotide exchange factors (GEFs)

All GEFs for Arfs share a common catalytic domain: the SEC7 domain, a module of approximately 200 amino acids that is sufficient to catalyze exchange of GDP for GTP on Arf in vitro [97]. Several GEFs have been identified in mammals, in yeast and in plant [29] and are classified in five subfamilies: Gea/Gnom/GBF, Sec7/BIG, ARNO/cytohesin/GRP, EFA6, and Arf-GEP100 [29,98]. The high molecular weight Arf GEFs of the Gea/Gnom/GBF and SEC/BIG subfamilies function in the ER–Golgi system, whereas the ARNO/cytohesin/GRP and EFA6 subfamilies function primarily in the endosomal-PM system [28]. Intriguingly, only two of these families, Gea/GBF/GNOM and Sec7/BIG, exist in *Arabidopsis*. The "large" GEFs are sensitive to brefeldin A, a fungal metabolite that induces the fusion of the Golgi apparatus with the ER, and acts by stabilizing an abortive complex between Arf1-GDP and the Sec7 domain of GEFs [99]. The structure of a complex between a truncated Arf1 (Δ17Arf1) and the Sec7 domain of Gea2 has been resolved [34]. The Sec7 domain catalyzes the GDP–GTP exchange by destabilizing the nucleotide with a glutamate residue (glutamic finger) and by restructuring the switch regions and the phosphate-binding loop of Arf1 [34]. All Arf GEFs identified to date are soluble proteins that are peripherally associated with membranes, and most of the high molecular weight GEFs are localized to the Golgi apparatus in mammalian cells [100–102]. By using quantitative confocal microscopy, it has been recently demonstrated that GBF1 colocalizes with the *cis*-marker p115, whereas BIGs overlap extensively with TGN38 [103]. This observation suggests that GBF1 and BIGs activate distinct subclasses of Arfs in specific locations to regulate different types of reaction. The GNOM ARF GEF of *Arabidopsis* has been shown recently to localize to endosomes where it controls the polarized trafficking of auxin efflux carrier PIN1 to the basal plasma membrane [104]. This result is different from yeast and animal cells where related GEFs are only associated with ER–Golgi secretory transport routes.

The low molecular weight subfamily of Arf GEFs are involved in signalling pathways downstream of PI 3-kinases, in actin cytoskeleton remodeling and in integrin signalling [88,105,106]. All ARNO/cytohesin/GRP family members contain a pleckstrin homology (PH) domain that mediates membrane localization via interaction with specific polyphosphoinositides, either phosphatidylinositol (3,4,5) triphosphate or phosphatidylinositol (4,5) diphosphate, and an adjacent carboxy terminal polybasic domain that cooperates with the PH domain to enhance membrane binding [28]. These small molecular weight GEFs (ARNO type) have not been reported in plants [107].

5. GTPase-activating proteins (GAPs)

GAPs stimulate the hydrolysis of bound GTP and regulates the Arf activity in the cell. The timing of GAP activity is critical for the function of GTPases, and in some cases, GAPs can participate in effector functions. Two types of Arf GAPs have been identified: small BFA-sensitive GAPs and large GAPs [108–112]. Both families of Arf GAPs contain a small catalytic zinc-finger domain that is sufficient to catalyze GTP hydrolysis on Arf. The consensus sequence of zinc-finger domain has four cysteine (NxxCxxCxxxxPxWxxxxxGxxxCxxCxGxHR where x indicates non-conserved amino acids) required for catalysis [35]. Several Arf-GAP proteins for mammals, yeast and plant Arfs have been identified and they all show a characteristic zinc finger motif [28,65,66]. In addition to

zinc finger, all Arf GAPs have a conserved arginine within the GAP domain. This arginine finger is essential to promote the hydrolysis of the third phosphate of GTP. Mutation of this arginine to lysine results in a 100,000-fold decrease in GTPase activity for ASAP1 [113] and for its close relative PAPβ [114]. Both GAPs seem to localize in the periphery of mammalian cells. Recently, a number of multi-domain proteins with an Arf-GAP have been identified and these are capable of interacting with proteins involved in both cell adhesion and actin organization [115]. Three new Arf-GAPs from the large GAPs family, ARAP1, ARAP2, and ARAP3, have been shown to contain both Arf-GAP and Rho-GAP domains [116,117], providing the first direct link between membrane traffic and actin cytoskeleton dynamics. These findings clearly indicate the dynamic nature of the mechanisms in which the Arf-GAP complexes are implicated, and it is not surprising that their regulation within the cell appears to be extremely complicated.

The structure of a complex between Arf1-GDP and the catalytic domain of Arf1-GAP has been crystallized [35]. It shows that unlike most GTP-binding proteins, the GAP binding site on Arf1 does not overlap with its effector binding site, the region of the molecule that interacts with coatomer in the GTP-bound state. Additionally, in contrast to previously characterized GAPs, Arf1-GAP does not supply any catalytic residues into the active site. In this case, it might be possible that Arf1 is simultaneously engaged in tripartite interaction with Arf1-GAP and coatomer, raising the possibility that coatomer might itself participate in the hydrolysis reaction by providing catalytic residue [35]. Indeed, when coatomer is added to Arf-GTP and the GAP, the GTPase reaction in vitro is accelerated 1000-fold [35]. Recent observations provide a convincing evidence for GAP-mediated hydrolysis of GTP-bound to Arf1 in either coatomer [118] or alternative effector complexes (GGAs) [119] with the concomitant release of Arf1-GDP to the cytoplasm. Another possibility of GAP and Arf1 interaction can be envisaged where Arf-GAP complex provides the binding site for the coat proteins and once the coat is formed and becomes stable, GAP hydrolyzes GTP and Arf in GDP form leaves the coat for recyling more rounds of coatomer addition [120]. This continuous activity of coatomer and Arf1 generates a kinetically stable membrane domains which ultimately would be transformed into either small coated vesicles or larger transport intermediates.

As in animal cells, plant Arf-GAPs have diversified into large family of proteins. *Arabidopsis* contains 15 proteins with Arf-GAP domains and are grouped into four distinct classes based on phylogenetic analysis and overall domain organization [66]. Class 1 (AtAGD1–AtAGD4 AtAGD3-*A. thaliana* Arf-GAP domain) contains a novel, plant-specific family of putative Arf-GAP proteins having pleckstrin homology (PH) domain, and two or three ankyrin repeat domains in addition to Arf-GAP domain (AGD). In addition, AtAGD1, AtAGD2, andAtAGD3 contain amino-terminal Bin1-am-phiphysin-Rvs167p/Rvs161p (BAR) domains and appear to be plant-specific. Class 2 AtArf-GAPs (AtAGD5–AtAGD10) show similarity both at sequence level and in overall domain organization to Golgi-localized Arf-GAPs in mammals and in yeast [107,121]. Class 3 AtArf-GAPs (AtAGD11–AtAGD13) are distinguished by the presence of a centrally located C2 domains, bind a range of ligands, such as phospholipids, phosphoinositides, and other proteins, in a calcium-dependent fashion [66]. Class 4 proteins (AtAGD14 and AtAGD15) could be integral membrane GAPs. Several AtArf-GAPs contain additional domains, and these may act to coordinate the timing of GAP activity within the cell or may serve functions beyond simply acting as negative regulators of Arf.

6. Arf-mediated recruitment of COPI proteins

The coat of COP I-coated vesicles consists of a heptameric coatomer protein complex (α-, β-, β′-, γ-, δ-, ε-, ζ-COPs), Arf1, and p24 family of integral membrane proteins [122–125]. Coatomer and GDP-bound Arf1 are soluble cytosolic factors that are recruited to Golgi membranes in a GTP-dependent manner [126,127], and this initiates COPI-dependent vesicle budding [1]. Once assembled, coatomer is a stable complex with a half-life of ~28 h in mammalian cells without exchange of subunits [128]. The β/δ COP heterodimer can bind to Golgi membranes in an Arf and GTP-γ-S dependent manner [129] and γ-COP subunit binds the cytoplasmic tail peptide of p23 (Ref. [130], see details in Section 8). A complete round of COPI coat assembly and disassembly has been reconstituted with purified components defining the core machinery of COPI vesicle biogenesis [30].

7. Mechanism of Arf1 activation and cargo selection

Upon activation by a nucleotide exchange factor [97,102,131–135], cytosolic Arf1-GDP is converted into membrane-bound Arf1-GTP [34,63,122,136–138]. This, in turn, triggers membrane recruitment of coatomer [126,127,139]. Direct interactions between Arf1 and coatomer have been reported and were shown to be GTP-dependent [140,141]. While only Arf1-GTP is stably associated with the membrane [34,63,122,136–138], Arf1-GDP can associate with membranes as well. Biochemical studies established that membrane recruitment of Arf1 must take place as a prerequisite for nucleotide exchange to proceed [142]. In addition, it has been shown recently that before binding of Arf in GTP form to the membrane, it first interacts directly to the cytoplasmic domain of p23 cargo receptor in GDP form [143]. These results establish a specific binding to the Golgi of Arf1-GDP as the first step of Arf1 recruitment and identify the cytoplasmic domain of p23 as a receptor for Arf1-GDP.

Several reports indicate that GTP hydrolysis by Arf1 is required for efficient cargo sorting and uptake into COPI vesicles, suggesting that the Arf1 GTPase cycle may allow for cargo selection and loading during COPI budding [144,145]. For example, cargo uptake is impaired in the presence of GTPγS or Arf-Q71L mutant protein [6,145]. It has been suggested that GDP/GTP exchange cycles and GTP hydrolysis open a time window that allows cargo to diffuse into budding zone. In this context, it is to be noted that coatomer has been reported to potentially stimulate Arf-GAP activity [35].

8. Arf interaction with p24 family cargo receptors

Recently, a family of type I transmembrane proteins of 23 to 27 kDa, termed p24 proteins, has been implicated in the formation of vesicles and the selection of cargo in both directions between the ER and the Golgi apparatus [146]. These proteins have a luminal domain of about 180 amino acids and a single membrane-spanning domain with a short (10 to 15 amino acids) cytosolic tail [147–149]. The members of all p24 proteins share common structural features such as double lysine or double arginine residues in their cytoplasmic tails [150]. A conserved feature is an additional diphenylalanine motif that forms a direct interaction with coatomers [151].

Currently, six family members have been identified in mammalian cells, whereas eight are known in yeast [150]. All p24 proteins are found in membranes of early secretory pathway [148,151–154], and there is evidence that they cycle constitutively between these membranes [153–155]. In Golgi membranes, p23, p24, and other members of the p24 family form hetero-oligomeric complexes [148,155,156]. The coiled-coil structures may promote association between identical or different members of p24 proteins, like Emp24p and Erv25p in yeast [157], or between p23 and p24 in mammals [152,154]. Moreover, overexpression of a single p24 protein leads to a mislocalization of all p24 proteins in ER-derived structures [152–154], and only the simultaneous overexpression of p24 proteins of all subfamilies results in a convincing perinuclear Golgi localization [152,153]. Furthermore, in yeast cells lacking one p24 protein [149] and in cells from mice lacking one allele of p23 [158], other family members are degraded. Thus, there is a strict dependence among p24 proteins in terms of stability, transport, and/or localization. Consequently, the formation of hetero-oligomers seems to be a prerequisite for their correct function. Recently, it has been demonstrated that p24 proteins are present in unequal concentrations and localize to differing extents to membranes of ER, IC (intermediate compartment), and Golgi, which excludes a simple 1:1 stoichiometry [159]. Moreover, individual members of these proteins exist as dimers or monomers and that the ratio between these two forms depends on both the organelle investigated and the p24 proteins. These studies point to a highly dynamic and complex system of altering dimerizations of p24 family proteins in the cell. These proteins not only form oligomers but are also highly enriched in early biosynthetic membranes, with p23 alone accounting for around 12,500 copies in membrane domains at the *cis* side of the Golgi complex [160]. In addition, p24 family proteins may be directly involved in protein and lipid sorting. A cholesterol-exclusion mechanism may well operate in the Golgi complex similar to that observed at the plasma membrane and in endosomes [161]. Recent data show that p25 and other p24 family proteins control the fidelity of membrane transport by maintaining cholesterol-poor membranes in the Golgi complex [161]. Similar observations were also reported with COPI vesicles which contain large amount of p24 proteins but are devoid of cholesterol [162].

On the basis of cross-linking studies as well as Arf1-binding experiments employing native Golgi membranes (mammalian origin), p23 proteins (a member of p24 family cargo receptors) were shown to play an important role in COPI coat assembly [125,151] and recently it was identified as Arf1-GDP receptor [143]. The identification of p23 as an Arf-GDP receptor [143] is consistent with recent in vivo studies demonstrating energy transfer between p23-CFP and Arf-YFP in living cells, an interaction detected only under conditions that allow Arf-mediated GTP hydrolysis [163]. Peptide-mapping studies revealed that the p23-interacting domain is located within the C-terminal 22 residues of Arf1 [143]. The available data in mammalian cells support the view that p23 and p24 are necessary requirement for COPI recruitment and coat assembly under physiological condition [158]. However, this view could be different in yeast cells where knockouts of all known p24 proteins did not affect the morphology of normal endomembrane system [164].

9. Minimal machinery for forming a COPI transport vesicle

The basic machinery for the formation of COPI-coated vesicles has been identified and requires well-defined liposomes and three protein components: Arf-GTPase, GTP, and cytoplasmic domains of putative cargo receptors (p23/p24 proteins) or membrane cargo proteins containing the KKXX retrieval signal projecting from the membrane bilayer surface [125]. Several groups have reported defined lipid compositions of liposomes that promote binding of various kinds of coat proteins resulting in vesicle budding and fission [37,38,165–167]. Bremser et al. [125], however, have recently shown that vesicle formation from liposomes composed of only egg yolk phosphatidylcholine (PC) is sufficient for COPI vesicle formation, provided that p23 lipopeptides are present. It suggests that specific lipids are not essential for COPI recruitment to membranes. However, it is highly unlikely that such lipid composition exists in a

biological membrane. While it is certainly possible that specific lipids (for example acidic lipids) might influence the rate of vesicle budding under physiological conditions in vivo [168,169], they do not appear to be essential components of the core machinery required for the formation of COPI vesicles.

10. A model for Arf1-mediated COPI-coated vesicle formation

The review of the literature outline above indicates that the minimal requirements for COPI-coated vesicle formation are heptameric cytosolic coat proteins, Arf1 and p24 family membrane proteins. A model to illustrate early steps of Arf1 recruitment to membranes is depicted in Fig. 2. Our recently published data [143] and the earlier findings of Beraud-Dufour et al. [62] demonstrate that Arf1-GDP is recruited to the membrane before nucleotide exchange. The binding of Arf1-GDP to purified Golgi membranes is virtually abolished in the presence of dimeric form of p23, indicating that an interaction between Arf1 and p23 at the membrane is necessary for efficient recruitment of the GTPase [143]. This interaction is likely to promote the activation of Arf1 by a nucleotide exchange factor [134,135]. This GDP–GTP exchange results in a change in conformational state of Arf1, which leads to its dissociation from p23 [15,31,32,34]. Our recent observations suggest that nucleotide exchange and Arf1 release from p23 are concerted events. As a result, the membrane becomes primed for coatomer recruitment both by membrane-associated Arf1-GTP (which interacts with β- and γ-COP; [140,141]) and, in close proximity, by p23 cytoplasmic tails (which interact with γ-COP; [130]). It is also possible that p23-mediated Arf1-GDP binding and subsequent nucleotide exchange modulate the oligomeric status of the complex of p24 proteins in order to generate coatomer-binding sites [170]. Arf-mediated GTP hydrolysis is differentially affected by members of the p24 protein family and this is implicated in their sorting into distinct classes of COPI vesicles [118]. Thus, it is likely that Arf1-GDP is produced continuously during budding process and some mechanism must efficiently retain Arf1-GDP in this budding zone for recycling. A mechanism to prevent the release of Arf1-GDP from membranes during early stages of coat recruitment would enhance the assembly of a pre-budding complex [143]. Coat polymerization then proceeds resulting in the formation of a coated bud, driven by the curvature of the assembling coat [171] (see details in Section 11).

11. Arf1-mediated COPI coatomer polymerization, budding and uncoating

Coatomer, in conjunction with the GTP binding protein Arf1 and p24 membrane receptors, forms an electron-dense coat that, when assembled onto Golgi membranes, is thought to drive membrane deformation, budding and eventually fission events associated to Golgi membrane traffic [17,170]. How could binding of coatomer complexes to the membranes induce membrane curvature? In vitro binding studies of coatomer and the cytoplasmic tail peptide of p23 suggest that this peptide binds to coatomer in an oligomeric state. In addition, this tetrameric interaction induces a conformational change in coatomer, possibly causing polymerization of coatomer complex [172]. Using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) and nanoESI-Tandem-MS, direct evidence for the spontaneous oligomerization of the cytoplasmic domain of p23 and p24 and their relative stabilities in solution was obtained. Coatomer is able to bind to such peptide oligomers in solution without any additional component added [156]. This raises the question as to why coatomer does not spontaneously bind to donor Golgi membranes containing ample amounts of p24-family proteins: only after membrane-association of Arf-GTP, coatomer can bind to Golgi membranes. Two alternative mechanisms may explain this strict Arf-GTP dependence: first, the p24 family recep-

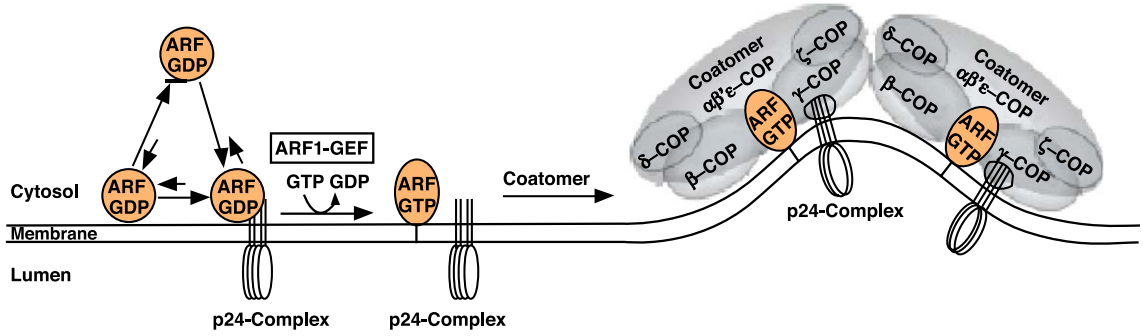

Fig. 2. A model shows the core machinery for the recruitment of Arf1 and coatomers to Golgi membranes. Arf-GDP binds to membrane phospholipids at low affinity. Upon binding to a p23 oligomer, this interaction is stabilized. Subsequently, a nucleotide exchange factor acts on Arf1-GDP and the resulting Arf1-GTP released from p23 receptor. Two binding sites of coatomer have now been generated (membrane-bound Arf1-GTP and a p23 oligomer) and this interaction induces a conformational change and polymerization of the complex, which shapes the membrane into a coated bud. (Reproduced from Gommel et al. [143] with permission from authors.)

tors may reside in the non-activated Golgi in non-homo-oligomerized forms but possibly in heterologous complexes [148,153,154]. Arf-GTP binding to the donor membrane could release individual p24 proteins to enable them to oligomerize and thus to create a productive binding site for coatomer. Second, Arf-GTP may regulate the thermodynamic equilibrium of coatomer between a soluble and a membrane-associated form by providing an additional binding-site to coatomer via its β- and γ-subunit [140,141].

Although previous results demonstrated a requirement for Arf-dependent GTP hydrolysis for uncoating COPI vesicles [173] but recent reports show that only catalytic domain of Arf-GAP is sufficient to initiate uncoating of liposomes-derived COPI coated vesicles [30]. This is also consistent with Goldberg's finding that the formation of tripartite complex of Arf1, GAP and coatomer is necessary for Arf1-GTP hydrolysis [35]. However, it is still not clear how Arf-mediated GTP hydrolysis controls the assembly and disassembly of the coats. For example, what is the precise time for Arf-GTP hydrolysis during coatomer formation? How does membrane curvature influence the rate of coat disassembly and how quickly does the coat dissociate after a vesicle has pinched off? Recently, Bigay et al. [174] put forth a promising model for COPI dynamics in which GTP hydrolysis in Arf1 is organized temporally and spatially according to the changes in lipid packing induced by the coat. Using time-resolved assays for COPI dynamics on liposomes of controlled size, they show that the Arf1 inactivation occurred more quickly on small liposomes (having high membrane curvature) than on large ones (with low membrane curvature). Furthermore, the stimulation of ArfGAP1 activity was greatest at radii approaching that of a typical coated vesicle, about 35 nm. This remarkable sensitivity of ArfGAP1 to membrane curvature could serve as a sensor for controlling the timing of Arf1-GTP hydrolysis [174,175]. This model provides a promising framework for explaining the possibility of bud formation through a continuous, self-regulating mechanism.

12. Vesicle trafficking in plants

Golgi apparatus in plants is the key organelle for the synthesis of complex cell wall polysaccharides and glycolipids. It plays a very important role in sorting proteins and sending them to their various destinations within the cell [52]. The plant Golgi apparatus shares many features with its animal counterpart, but also has unique characteristics. The most significant difference concerns its structure. Whereas in animal cells the Golgi apparatus occupies a rather stationary perinuclear position, in plant cells it is organized into a large number of independent stacks that are distributed throughout the cytoplasm and can move along actin filaments [52,176,177]. These stacks are generally considered to be functionally independent. This dynamic localization likely requires different transport mechanisms both to and from the Golgi when compared to the well-studied mammalian systems. Furthermore, plant Golgi stacks, in contrast to mammalian cells, do not disassemble during mitosis and one of the prominent features of the plant Golgi apparatus is the synthesis of polysaccharides, and these are required in large amounts for cell plate formation during cytokinesis [178,179]. The important role of the Golgi in producing cell wall material [44] put different constraints on the flow material through stacks. The GFP studies have led to formulation of a variety of models to account for the membrane traffic between the ER and Golgi apparatus in plants. One proposal is that the rapid movement of Golgi stacks along the actin filaments and their close association with ER allow the Golgi to continually collect vesicles budding from the ER (namely the vacuum cleaner model) [180]. This model suggests that Golgi stacks move over the surface of the ER to collect the vesicles, which bud from the ER. This implies that the whole surface of the ER is able to export protein and Golgi movement is essential for efficient targeting and consumption of transport vesicles. In contrast, a second model attaches particular significance to the alteration between the vectorial and oscillatory modes of Golgi movement [177]. In this stop-and-go model, cargo collection would occur only when Golgi stacks stop at ER exit sites, which produce a local stop signal that transiently halts stack movement. A third model proposes that Golgi movement represents the movement of both ER and Golgi stacks that behave as one dynamic system, either through direct membrane continuities or through continuous vesicle formation/fusion reactions. In this model, Golgi stacks and ER export sites may move together in an actin-dependent fashion, forming discrete 'secretory units' [50]. These findings and the interesting models which have been put forward have led to an intense interest in characterizing ER/Golgi trafficking in plant cells (also see Sections 13, 14 and 20). In vivo observations of membrane proteins from ER to Golgi have recently been facilitated by the use of fluorescent protein chimeras. Selective photobleaching experiments using two fluorescent marker proteins locating to the Golgi (ST-YFP and ERD2-GFP) have allowed confocal imaging of ER-to-Golgi protein transport [51].

There are a number of other features that are unique to plant secretory pathway. For example, plant cells possess a unique and complex vacuolar targeting machinery. This complexity is possibly due to the existence of two distinct populations of vacuoles in some cell types [181–183]. Presumably one type of vacuoles possesses a lytic function and the other one possesses a storage function. Both appear to receive soluble and membrane proteins from the Golgi. Recent studies on vacuolar targeting have identified two critical molecular components of sorting machinery, which partially overlap in their subcellular location [184,185]. This multiple targeting at vacuolar compartments in plant cells requires sorting and targeting mechanisms that differ from their counter part in yeast or animal cells [186–190]. The *Arabidopsis* genome contains a remarkable number of

SNAREs, which are known to play an important role in the fusion of transport vesicles with specific organelles. They are one of the essential parts of vesicle targeting and fusion machinery in mammalian and yeast cells [8,10,107,187]. There are 24 genes found in *Arabidopsis* genome which share homology to the syntaxin family of SNAREs [188]. Several of them can complement corresponding yeast vacuolar targeting mutant [191]. This large number of SNAREs in *Arabidopsis* genome is unprecedented. Human or worms or flies have a much less number of SNAREs than plants although their genome is much larger than *Arabidopsis*. Despite the presence of a large number of gene families in plants, each syntaxin has a unique essential function as indicated by the lethality of gene disruptions in individual syntaxin genes. At least 20 of these genes are expressed in plant cells and therefore unlikely to be pseudogenes [188,192]. It appears that some of these proteins have specialized in the plant-specific cytokinesis [193–195], whereas others appear to have taken on a role in hormone signalling [196–198]. Since it is known that protein targeting in plant cells can be polarized to particular domains of the plasma membrane [199], it is possible that SNAREs have specialized into these roles [188,200].

The proteins that mediate export from the ER (COPII coats, Sar1p and its GEF Sec12) are present in plant cells (for details see Section 20). The components of COPI machinery have been located to the Golgi by means of immunofluorescence in tobacco BY-2 cells [48] (see also Sections 13 and 14). Moreover, a study combining biochemistry and immunocytochemistry techniques revealed precise localization of the COPI components and Arf1p in plant cells [201]. In addition, the presence of calreticulin and HDEL-tagged α-amylase in plant COPI vesicles indicates that like in mammalian cells, these vesicles might be involved in retrograde transport from the Golgi [202]. The involvement of AtArf1 in this particular transport event was also deduced from the effect of two dominant negative mutant forms of AtArf1 on the localization of a *cis*-Golgi marker, AtErd2-GFP, from the Golgi apparatus to ER [203].

Similar to its mammalian counterpart, AtRab1 (*A. thaliana*) and NtRab2 (*Nicotina tabacum*) seems to be

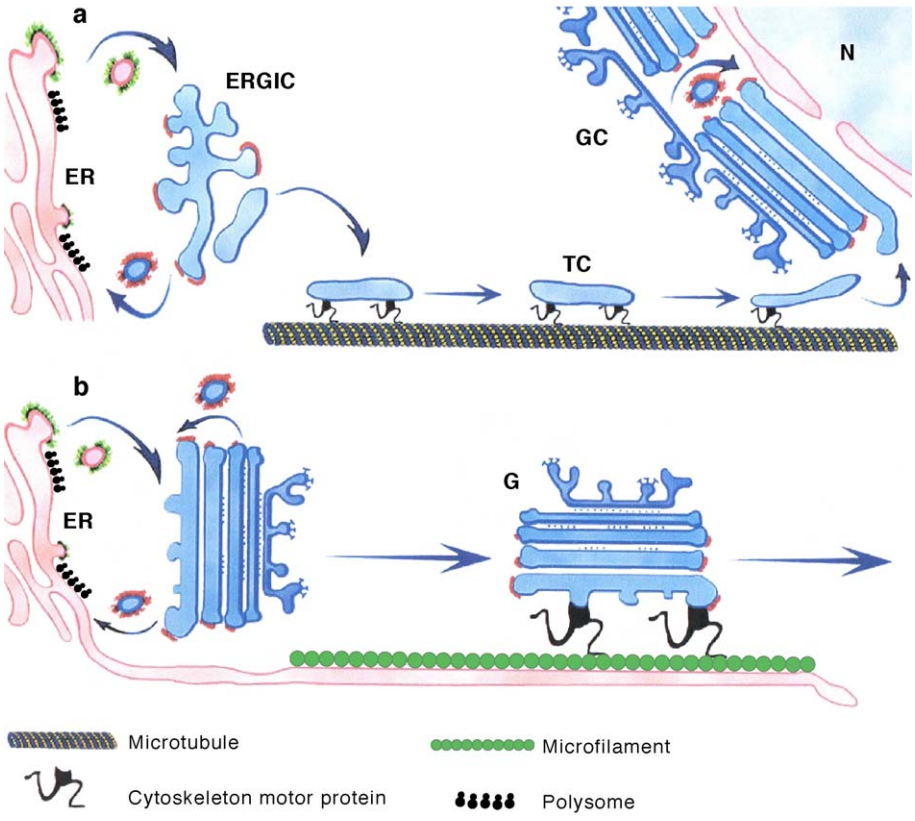

Fig. 3. Comparison of ER–Golgi trafficking in mammalian and plant cells. In mammalian cells (a), COPII vesicles bud from ER and fuse with ERGIC (ER–Golgi intermediate compartment). The escaped proteins and COPII machinery proteins recycle back from ERGIC to ER through retrograde COPI vesicles. The remaining proteins travel either through vesicles or through transport containers (TC), which move along microtubules to the perinuclear Golgi complex (GC) where they form new *cis*-cisternae. In plant cells (b), because of the nonexistence of ERGIC, COPII vesicles directly fuse at the *cis*-Golgi of individual stacks. Long-distance transport is achieved by active movement of intact Golgi stacks (G) along actin filaments. Retrograde transport from the Golgi to ER possibly takes place either near ER export sites or randomly during translational movement of Golgi stacks. (Reproduced from Nebenfuhr et al. [208] with permission from authors.)

involved in ER–Golgi transport [204,205]. A dominant negative mutant, AtRab1b (N121I) inhibited the secretion of a fluorescent marker protein (sec-GFP) as well as the Golgi localization of a Golgi-targeted fluorescent marker (N-ST-GFP), leading to fluorescence accumulating in the ER in both cases. Green fluorescent-NtRab2 fusion protein localized to the Golgi bodies in elongating pollen tubes but did not target efficiently in the Golgi bodies of leaf protoplast and epidermal, suggesting that it plays an accessory role in cells that have very active secretory system [205].

Recently, the primary target of BFA in plant cells has been identified [48]. While in essence confirming that the primary target of BFA in plants is a Golgi localized Arf-GEF (similarly in animal cells), these studies also provided an important evidence for cisternal maturation as a means of anterograde intra-Golgi transport. In addition, through the formation of unique BFA-induced ER–Golgi hybrid structures, they underline the fundamentally different plant ER/Golgi transport relationship. Briefly the main differences in ER–Golgi trafficking in animal and plant cells can be described as follows: In mammalian cells, COPII vesicles (see also Sections 17 and 18) fuse with the ER–Golgi intermediate compartment (ERGIC) (or vesicular-tubular cluster, VTC) (see Fig. 3). These compartments represent the first site of segregation of anterograde and retrograde protein transport [206,207]. In plant cells, COPII vesicles directly fuse at the *cis*-Golgi of individual stacks because the ERGIC does not exist. Retrograde transport from the Golgi to the ER may occur near ER export sites or randomly during translational movements of Golgi stacks. Plant Golgi stacks move along actin microfilaments, which run parallel to the ER unlike the situation in mammalian cells (Fig. 3) [52,208]. These studies indicate that although plant cells use essentially the same machinery for vesicular trafficking as yeast or mammalian cells, they have adapted several of these proteins to the unique requirements for their secretory system by using them for transport between membrane compartments not found in other organisms.

13. Plant Arf1p and p24-family homologues

The cDNAs of *Arf1* with high sequence similarity to mammalian Arf have been isolated from *Arabidopsis* [209], the green algae *Chlamydomonas reinhardtii* [210], and several crop plants (carrots, [211]; maize, [212]; potatoes, [213]; rice, [214]). The *Arabidopsis* genome contains 18 *Arf* genes with 12 isoforms present in *Arf* and 6 isoforms in *Arl* subfamilies [66]. Six AtArf GTPases cosegregated with human Arf1 and Arf3 sequences and one of them was reported to be localized (AtArf1) to peripheral Golgi stacks along with an *Arabidopsis* homologue of the COPI coat protein complex [48,209]. Little is known about the function of Arl GTPases although one member provides an essential function in membrane trafficking steps necessary for proper cell deposition during cytokinesis in developing embryo [215]. The mutation in the *TITAN5* gene, which corresponds to *AtArlC,* exhibited dramatic alterations in mitosis and cell cycle control during seed development in *Arabidopsis.* Rice and wheat *Arf1* are completely identical at the DNA level [214,216]. Genomic Southern hybridization indicates that wheat *Arf* is encoded by at least two or three copies of *Arf1* genes. Moreover, it seems that *Arf* transcription and translation is greater in roots than in shoots [216]. Arf1 has been identified in pea plumules by Western blotting; it is recognized by an antibody prepared against mammalian Arf1 antibody [217]. In *Chlamydomonas* there is a biphasic pattern of Arf1 mRNA accumulation during the light/dark growth cycles, which is also reflected at the protein level [210]. *Arabidopsis* AtArf1 is shown to complement yeast *arf1 arf2* mutants and its GFP-fusion is localized to the Golgi apparatus in plant cells like its animal counterpart [203] and plays an important role in the intracellular trafficking of cargo proteins and functions through a brefeldin A (BFA)-sensitive factor in *Arabidopsis* [218]. Plant cells contain two different types of vacuoles: a lytic vacuole akin to those in yeast or the lysosome of animal cells and a large storage vacuole [219], and recent findings suggest that Arf1p is also involved in the BP-80-mediated transport route to the lytic vacuole [202].

All reported Arf-GEFs posses a Sec7 domain (see above), a 200-amino-acid domain which is sufficient to exchange GDP for GTP on Arf [29]. Eight genes encoding *Arabidopsis* GEFs have been reported [66] and one of them was identified as GNOM in mutant screens for *Arabidopsis* with defective body organization in embryos [220]. This GNOM mutant is defective in the transport of auxin, through mislocalization of auxin efflux carriers of the PIN1 type [199,221]. A similar phenotype is caused by brefeldin A treatment. It turns out that the GNOM mutation lies in an allele for Arf-GEF. When BFA is removed, the normal localization is restored rapidly [221,222], suggesting that this vesicle-dependent targeting is dynamic in character. Recently, Geldner et al. [104] have demonstrated that GNOM localizes to endosomes where it controls the polarized trafficking of PIN1 to the basal plasma membrane. This response of high molecular weight GEFs (GNOM) in plants is different from that in mammalian cells where large GEFs are generally involved in ER–Golgi trafficking [28]. The function of GNOM in PIN1 sorting is likely mediated by activation of one or more of the six *Arabidopsis* Arfs. The activated Arfs in complex with GNOM could subsequently recruit to endosomes a protein coat involved in PIN1 sorting. By analogy with mammalian cells, the *Arabidopsis* homologues of AP-1 and AP-4 are particularly interesting candidates to play such a role. Little information is available about the function of other *Arabidopsis* Arf-GEFs. However, it appears that Arf-GEFs are one of the key components of vesicular trafficking and could play an important role in plant growth and development.

Arf-GAPs, which are involved in GTP hydrolysis of Arf, are multidomain proteins containing either a zinc finger (GAP) domain or both GAP and pleckstrin homology (PH) domains (see Section 5). *Arabidopsis* contains 15 Arf-GAP domain proteins (AGD) and are classified in four groups. Three groups are related to yeast Arf-GAPs acting at the TGN, Age2p, Gcs1p and Glo3p, respectively, while the members of the fourth group show similarities with human Arf-GAP1 [66,223].

The p24 family membrane proteins also exist in plants, but the low number of available sequences precludes any thorough analysis at this stage. Data bank analysis of the *Arabidopsis* genome reveals about eight p23-like sequences, but nothing is known about their expression, localization or function (Memon, A.R., unpublished data). These results indicate that the molecular machinery needed for both anterograde and retrograde vesicle transport between ER and Golgi exists in plants and is mediated by the same set of proteins as in animal and yeast cells.

14. Arf1-mediated COPI-coated vesicle formation in plant cells

Sequence databases have been used to search for potential plant homologues of the characterized yeast and mammalian proteins [49,224], including the components of the coats of COPI vesicles and the carriers participating in the transport between ER and Golgi. Although many potential plant homologues of the proteins involved in anterograde and retrograde transport have been found [49], very few component proteins have been identified experimentally. Several putative COPI subunits have been identified in rice cells by using several antibodies raised against mammalian COPI proteins [225].

Evidence is accumulating for COPI homologues playing similar roles in plant cells, but this is neither well characterized nor well understood [42,201,226]. Pimpl et al. [201] showed in situ localization of COPI-coated vesicles and demonstrated in vitro the recruitment of coatomer, the protein complex that makes up the COPI vesicle coat, from a cytosolic fraction onto budding vesicles. Antisera prepared against recombinant plant COP-coat protein homologues have allowed for the identification of cytosolic protein complexes similar to coatomer and the Sec23/24 dimer from mammalian and yeast cells [226]. These and additional antisera against Arf1p/Sar1p homologues have successfully been employed in immunogold electron microscopy to localize plant Arf1p and coatomer to budding vesicles at the periphery of *cis*-cisternae in *Arabidopsis* and maize root cells [201]. It has also been possible to recruit Arf1p and coatomer from cauliflower cytosol onto mixed ER/Golgi membranes, as well as to provide evidence for the release of plant COPI-vesicles in vitro [201].

In mammalian and yeast cells, COPI-coat formation is triggered by the conversion of Arf1 in its GDP-form to a GTP-form, a process which can be inhibited by brefeldin A [227], leading to the release of membrane bound coatomer [228]. It has recently been established that the primary effect of brefeldin A on plant cells is rapid displacement of coatomer into the cytosol, and is similar to that in other eukaryotes. The subsequent manifestation of this toxin on the endomembrane system is, however, very different [46,48,208]. It is interesting that the events lying between the BFA-induced loss of COPI coats from the Golgi on the one hand, and fusion of Golgi membranes with the ER on the other hand, are strikingly different between mammalian and plant cells [48,208]. These differences most likely reflect the different structural organization [229,230] and functional requirement [46,231] of the Golgi apparatus in two systems (see Fig. 3).

15. Sar1 GTP binding proteins

Sar1 defines a unique family within *Ras* superfamily of small GTPases and is significantly divergent from its closest relative, Arf1. It is 37% identical to class I Arfs and 41% identical to Arf6. Most families of small GTPases, including Ras, Rho, Rac and Rab, have cysteine residue(s) at their C-termini and are modified with lipidic moieties such as farnesyl or geranylgeranyl and Arf, a closest relative of Sar1, and commonly have a myristoyl modification at their N-terminus. In contrast, Sar1 has no posttranslational modifications either at the N- or C-terminus. Sar1 functions as a molecular switch to control the assembly of protein coats that direct vesicle budding from the ER [36,37,232]. The GTPase cycle of Sar1 and its interacting proteins have been studied extensively in yeast and its regulatory mechanism appears to be similar as proposed for Arf [17,39]. And this is supported by marked structural similarity of Arf1-GDP and Sar1-GDP, and of Arf1-GppNHp and Sar1-GppNHp [31,34,40,41] (see Figs. 1 and 4). A transmembrane protein of the ER, Sec12 acts as a GEF and catalyses the exchange of GTP for GDP on Sar1, allowing the latter to bind to the ER membrane and form the binding site for COPII coat proteins [39,233]. The three-dimensional structure of the Sar1 in its GDP-bound form showed a number of structurally unique features that dictate its biological function [40] (Fig. 4). It contains a conserved cluster of nine hydrophobic amino acids, referred to as the Sar1-NH_2-terminal activation recruitment motif (STAR), which mediates the recruitment of Sar1 to ER membranes and facilitates its interaction with Sec12 GEF leading to activation. In addition, an NH_2-terminal amphipathic $\alpha 1'$-helix of Sar1 facilitates its functional interaction with Sec23/24 GAP complex and is responsible for cargo selection during ER export [40]. It has been proposed that hydrophobic Sar1 NH_2-terminal activation/recruitment motif, in conjunction with the α 1′-helix, mediates the initial steps in COPII coat assembly for export from the ER [40]. The recent crystallographic work of Bi et al. [41] indicates that binding of GTP induces a

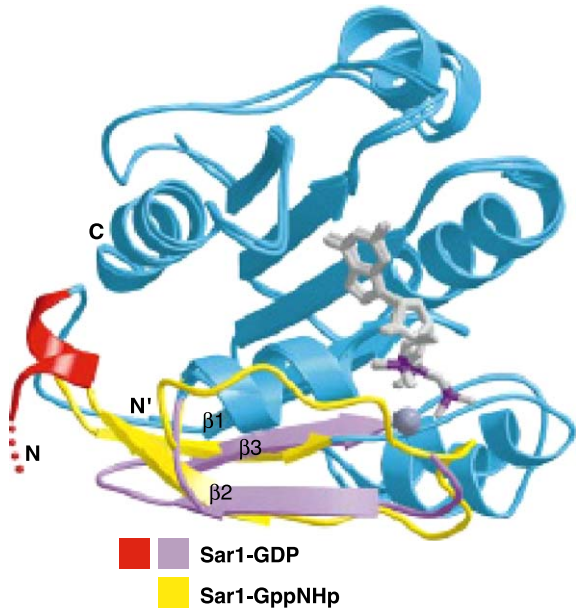

Fig. 4. Sar1 GDP-GTP conformational switching. Superimposed structures of mammalian Sar1-GDP and yeast Sar1-GppNHp suggest a GTP-induced translation of β-strands 2 and 3 from the diphosphate (pink) to the triphosphate (yellow) conformation. This releases the amino-terminal α-helix (red) for interaction with the lipid membrane. (Reproduced from Bi et al. [41] with permission from authors.)

conformational change within Sar1 that allows an amino-terminal extension and an amphipathic α-helix to engage in interactions with membrane phospholipids and for several loop regions to associate with Sec23 (Figs. 4 and 5).

16. SAR1-mediated recruitment of COPII proteins

Coat protein complex II (COPII)-coated vesicles shuttle diverse cargo molecules from exit sites of the ER towards the early Golgi complex [39,233,234]. Vesicle formation involves the stepwise recruitment of SAR1 GTPase, followed by two large heterodimeric complexes Sec23p–Sec24p (Sec23/24p) and Sec13p–Sec31p (Sec13/31p) to the membrane [39]. Isolated Sec23/24 and Sec13/31 complexes have been visualized by electron microscopy, and the atomic structure of Sec23/24 in complex with Sar1-GTP has been determined [41,235,236] (Fig. 5). Sec23/24 complex contains one Sec23 subunit (85-kDa) and one Sec24 subunit (104-kDa) [237] and Sec23 functions as a GAP for Sar1. Although Sec23 association with Sec24 is not required for Sec23 GAP activity, both proteins are required for vesicle formation [39,238]. The Sec13/31p complex comprises the proteins Sec13p (~ 33 kDa) and Sec31p (~ 140 kDa), both containing WD40 motifs [239]. These motifs are implicated in protein–protein interactions. Together these two proteins form a heterotetramer composed of two copies of each and form a 700-kDa heteromeric complex [239,240]. Moreover, the Sec23 GAP activity towards Sar1 is further stimulated when Sec23/24–Sar1 complexes are gathered by Sec13/31. This mode of GTPase regulation seems to provide COPII with a built-in disassembly program [241].

Deep-etch rotary shadowing and electron microscopy were used to explore the COPII subunit structure with isolated proteins and coated vesicle [236]. Sec23/24p resembles a bow tie, and Sec13/31p contains terminal bilobed globular structures bordering a central rod. The surface structure of COPII vesicles revealed a coat built with polygonal units. The length of the side of the hexagonal/pentagonal units is close to the dimension of the central rod-like segment of Sec13/31p. COPII complexes are therefore different from the clathrin triskelion. However, these proteins are able to form a coat, with an apparent lattice structure, on membranes, although a much less obvious one than that of the clathrin coats. This suggests a regular mode of assembly [236]. Using dynamic light scattering, it has been shown that Sec23/24 and Sec13/31 can self-assemble in a stoichiometric manner in solution to form particles with hydrodynamic radii in the range of 40–60 nm. Self-assembly is favoured by lowering the pH, the ionic strength and/or the temperature. Electron microscopy

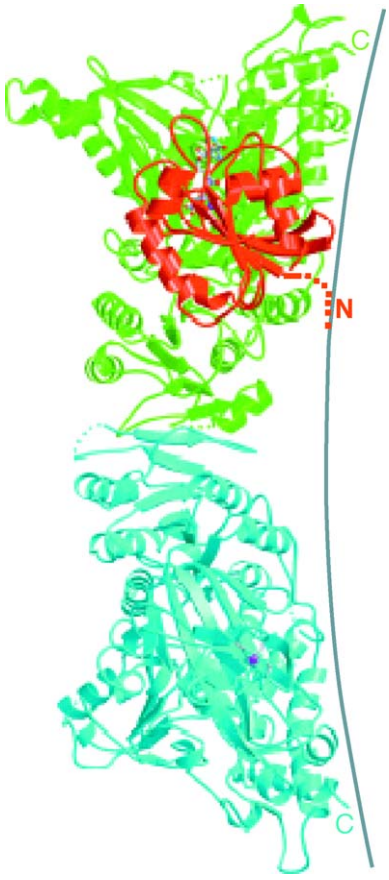

Fig. 5. Structure of the Sec23/24–Sar1 prebudding complex. Ribbon diagram of the crystal structure of the Sec23/Sec24–Sar1 complex. A gray curve line indicates the membrane surface of a 60-nm vesicle. Sec23, yellow; Sec24, green; Sar1, red. (Reproduced from Bi et al. [41] with permission.)

reveals the formation of spherical particles 60–120 nm in diameter with a tight, rough mesh on their surfaces that may mimic the polymerization state of COPII proteins on membranes [242].

17. Sar1-GTP-dependent cargo recruitment

Sar1 not only controls COPII budding, but also integrates coat assembly with cargo selection. Under conditions that restrict Sar1 to its active GTP-bound form, Sec23/24–Sar1 prebudding intermediates accumulate in complexes with protein cargo to be included in COPII vesicles [13,14,238,243]. This provides a mechanistic explanation for selective capture of cargo molecules into forming vesicles. For some cargo molecules, ER export motifs that act in COPII-dependent export have been defined [13,233,234]. In studies of vesicular stomatitis virus G protein (VSV-G) [244] and potassium channel proteins [245], it has been shown that these proteins contain a di-acidic DXE amino acid motif (where X can be any amino acid) near their carboxy terminal tail which is required for ER export. In the case of VSV-G, however, it appears that additional elements mediate COPII binding [246], and an overlapping tyrosine-based signal (YXXØ) has been identified that is required for optimal ER-export rates [247]. The di-acidic amino acid motif also functions in yeast Sys1p [248]. ER export motifs of other integral membrane proteins that cycle between the ER and Golgi compartments, such as p24 proteins and ERGIC-53, have been identified [148,249]. The export of these proteins depends on a pair of hydrophobic residues (e.g. FF, diphenylalanine or LL, di-leucine) contained in their cytoplasmically exposed tail sequences. Binding studies indicate that the attachment of these hydrophobic signals to reporter molecules accelerates their transport efficiency and it appears that the exact composition and position of the hydrophobic pair seem unimportant [250]. The mutation of FF motif in mammalian p24 tail sequences interfered with binding to the Sec 23 subunit of COPII and caused accumulation in the ER [148]. Similarly, the export of p24 complex in yeast also requires the presence of a pair of aromatic residues: FF on the Emp24p tail and YF (tyrosine–phenylalanine) on the Erv25p sequence [251]. Recent studies on Erv41p–Erv46p complex, which cycles between ER and Golgi, have identified multiple ER export signals contained within the C-terminal cytoplasmic tails of both Erv41p and Erv46p. Sequence information contained in these tails of both the Erv41p and the Erv46p is required in a specific orientation for efficient packaging of the Erv41p–Erv46p complex into COPII vesicle [252]. In some cases, cargo oligomerization has shown to be essential for its exit from ER [253]. For example, in yeast cells, Emp47p, a type-I membrane protein, is specifically required for the transport of an integral membrane protein, Emp46p, from the ER. Binding of these proteins occurs in the ER through the coiled-coil region in the luminal domains of both proteins and their dissociation occurs in the Golgi. This coiled-coil region is necessary for Emp47p protein to form an oligomeric complex that is essential for its export from the ER. The oligomeric association of other type-I transmembrane cargoes has also been reported for p24 family members, which also depends on the coiled-coil region of their luminal domain [149,254]. Although the precise role of cargo oligomerization in the ER export remains to be established, these findings suggest the presence of assembly-dependent cargo selection at the ER exit sites. Database searches of other transmembrane proteins that are exported from ER show that they often contain these hydrophobic motifs, and in some instances are known to be required for ER export [255,256].

In other cases, accessory factors are required to direct transmembrane secretory proteins into COPII vesicle [257]. For example, some integral membrane cargo may not possess adequate sorting signals for efficient ER export and will rely on COPII adaptors [258]. All this information on ER-export signals suggests that there may not be a single stringent code, but rather multiple signals exist that may be decoded by COPII budding machinery [233,234]. Recently, a site on Sec24 (a component of COPII coatomer) has been identified which recognizes the v-SNARE Bet1p and the mutations introduced at that site disrupted the packaging of a number of cargo molecules [13]. The same site was also shown to be conserved as a cargo interaction domain on the Sec24 homologue Lst1p, which only packages a subset of the cargoes recognized by Sec24p. These functional studies together with a detailed structural and biochemical analysis of coat-SNARE interaction [14] demonstrate that cargo recruitment into nascent vesicle is driven by direct interaction between a sorting signal and a coat subunit. This provides an important basis for identifying distinct sorting signals and novel cargo interaction domains.

18. COPII vesicle polymerization and budding

Studies of the cargo-selection function of Sec23/24–Sar1 in yeast and mammalian systems have shown that ER-to-Golgi SNAREs and at least some integral membrane cargo proteins can bind directly to the pre-budding complex [13,14,238,243,248,259,260], leading to their concentration in COPII vesicles [261]. A final function of Sec23/24 is to act as GAP on Sar1-GTP [262]. It seems that, as the prebudding complex forms, a slow GTP hydrolysis reaction is programmed into the assembling coat to limit its lifetime and to couple coating to uncoating [41,241]. Thus, as in COPI, an initial complex is formed between the incoming coat component (Sec23/24p), the GTPase Sar1 in its GTP-bound form and a membrane protein which will be subsequently included into the vesicles. These membrane proteins

have been proposed to work as "primers" that would be able to nucleate the COPII coat [17,259]. This would guarantee their packaging into COPII vesicles, linking the budding of a vesicle to the incorporation of proteins that are essential for its travel or fusion.

The molecular aspects of COPII function in ER export were recently addressed by determining the structure of the Sec23/24–Sar1 complex [41] (Fig. 5). Bi et al. [41] have assembled an atomic model of the Sec23/24–Sar1 pre-budding complex from the individually determined structures of Sec23–Sec24 and Sec23–Sar1 bound to nonhydrolyzable GTP analog GppNHp (Fig. 5). This crystal structure provides useful information about the cargo recognition by Sec23/24–Sar1 complexes. The overall structure of the complex reveals a bow-tie-shaped 15-nm-long assembly as described previously [236]. The elongated Sec23/Sec24–Sar1 complex largely extends parallel to the membrane bilayer, with a positively charged concave inner surface that could favour membrane deformation and perhaps exert some influence on the final dimension and rather uniform size of COPII vesicles that bud from purified ER membranes (Fig. 5). This chemical feature also explains the need for acidic phospholipids in budding COPII vesicles from synthetic liposomes [38,39]. The Sec23 and Sec24 exhibit closely related folds comprising five distinct domains: a β-barrel, a zinc finger, an α/β vWA or 'trunk' domain forming the dimer interface, a α-helical region, and a carboxy-terminal gelsolin module [41]. Gelsolin domain does not contribute to the inner surface of the coat; instead, it lies against the helical domain, forms critical catalytic interactions with Sar1 and the nucleotide, and extends radially away from the membrane, perhaps to form an interaction site for Sec13/31. Sec23 forms an extensive interface with Sar1-GTP contacting conserved residues contributed by three of the Sec23 domains. Given the variety of cargo that must be recognized by the COPII budding machinery, multiple regions of the Sec23/24 protein could be used for cargo selection. Additionally, it has been shown that distinct Sec24 family members pair with Sec23 and operate in cargo recognition to expand the range of cargo incorporated into prebudding complexes [13,39,41].

ER membranes with Sec23/24–Sar1 can then recruit Sec13/31 and as coat protein complexes on the membrane grow, other cargo proteins may diffuse into them and become captured by interaction with the coat. At this point, membrane or lumenal secretory proteins that cannot themselves interact with the vesicular coat may also become included in the vesicle budding site if they are recruited by a protein that binds to the coat. Finally, in the last stage, the polymerized coat deforms the membrane, and a vesicle buds off. No additional cytosolic or membrane proteins seem to be required for this step to occur in vitro from isolated donor membranes or defined liposomes [37,38], but whether and how this step is regulated in vivo remains unknown. Recently, Ypt1p-interacting protein (Yip 1p) has been implicated in COPII vesicle biogenesis [263]. Antibodies directed against the amino-terminal cytosolic domain of Yip1p potentially inhibited budding of COPII vesicles, and vesicle tethering and fusion were not significantly affected by the presence of these antibodies. Genetic interaction analyses of the yip1-4 mutation also corroborate a function in ER budding. Although a series of events in COPII loading and budding have been described, our molecular view of these processes remains to be established.

19. Sar1-GTP hydrolysis and coat disassembly

Nucleotide exchange and GTP hydrolysis on Sar1 are two important events, which must be spatially and temporally organized to coordinate coat assembly with cargo selection and coat disassembly with vesicle fission. At present, it is unclear at which time point during the budding process Sar1-GTP is hydrolyzed. Although GTP hydrolysis is required for the release of Sar1p from the vesicular membranes and the subsequent uncoating of the vesicles (to allow fusion with the target membrane), it could occur much earlier, possibly even before the vesicle separates from the donor membrane. This need not result in early disassembly of the vesicular coat: COPII vesicles that are produced from microsomes in the presence of GTP contain no Sar1, and yet they have a COPII coat that is visible in the electron microscope [264]. Thus, although GTP hydrolysis leads ultimately to coat disassembly, a coated structure may persist when some Sar1 molecules are lost. Once bound in a polymeric lattice, Sec23/24 and Sec13/31 may no longer need Sar1p to adhere to the vesicle; instead, their interaction with primer proteins may be sufficient. The fact that Sec23/24 and Sec13/31 can self-assemble in solution suggests that lateral contacts between these complexes may be strong enough to transiently preserve the structure of the coat, provided that the GTP hydrolysis reaction is properly organized in time and in space [39,242].

A 'two-gear' mechanism has been proposed for the control of GTP hydrolysis, on the basis of kinetic studies showing that the lifetime of the Sec23/24–Sar1-GTP complex is relatively long (~30s), and hydrolysis is accelerated about 10-fold after Sec13/31 binding. Hence, the COPII triggers its own disassembly, but the lifetime of GTP on Sar1 may be matched to the rate of budding to give prebudding complexes time to collect SNARE and cargo molecules, and completion of polymerization before disassembly [241]. Further inspection of the Sec23–Sar1 interface reveals the mechanism of Sar1 GTPase stimulation [41]. Sec23 furnishes an arginine "finger" to the Sar1 active site to form bonds to the phosphate groups through its guanidium side chain, thereby stablizing the transition state. This will facilitate the GTP hydrolysis on Sar1 and, hence, coat disassembly.

20. Sar1-GTP binding proteins in plant cells

Plant Sar1 has been identified from number of plant species [265–269]. Three Sar1p homologues have been identified in *Arabidopsis* [66]. Phylogenetic tree analysis showed their cosegregation with animal and yeast Sar1 GTPases and were renamed as *AtSarA1a* (previously identified as *AtSar1*), *AtSarA1b*, *AtSarA1c* (previously identified as *AtSar2*) [66]. *AtSarA1a* was shown to functionally complement mutants in *S. cerevisiae* [265] and *AtSarA1a* overexpression in transgenic plants enhanced its secretion activity from ER membranes to cytosol [267]. *AtSar1* and *AtSec12* (a 43-kDa GEF) are both associated with the ER. However, about one-half of the cellular *AtSar1p* was also found in the cytosol [267]. The intracellular localization of AtSar1 was modified by cold shock treatment, which reduced its association with ER membrane [267]. Stable overexpression of Sec12p in transgenic plants neither affected cell viability nor caused a redistribution of Sar1p [267]. In contrast, transient overexpression of Sec12p in tobacco protoplasts resulted in recruitment of the GTPase to the ER membrane [45] and resulted in a dosage-dependent inhibition of α-amylase secretion (a cargo molecule). Similar inhibition in secretion was also observed when tobacco protoplasts were overexpressed with dominant-negative GTP-trapped mutant of Sar1p (H74L) [45]. One likely explanation of these results would be that plant cells contain regulatory mechanisms to respond to an imbalance in the Sec12p/Sar1p ratio [45]. Under these conditions, Sec12p overexpression inhibits COPII transport through the depletion of Sar1p, therefore preventing the formation of COPII vesicles. Co-expression of a dominant negative mutant of Sar1p (H74L), which lacks the GTPase-activating activity of Sec23p, inhibits COPII transport at a later stage. Probably, it interferes with the uncoating and subsequent membrane fusion of the vesicles. The transient expression of AtSar1-H74L, a dominant negative mutant, blocked the transport of AtERd2 and AtRer1B (*cis*-Golgi membrane proteins) from ER to the Golgi apparatus in tobacco and *Arabidopsis* cells. The same mutant of Sar1 also blocked the exit from the ER of a vacuolar storage protein, sporamin [269]. Sar1 (H74L) and Sar1 (T34N) mutants efficiently blocked the ER to Golgi transport in *Nicotiana cleveland* leaf epidermal cells [270]. Similar but weaker effects were observed with antisense construct but not in the leaves transfected with sense construct. Leaf transfection with Sar1 (H74L) and Sar1 (T34N) mutants resulted in gradual cell death and leaf degeneration [270]. COPII-mediated anterograde transport has been recently examined in plants with all indications that it is similar to that found in yeast and mammalian cells. Several groups have cloned and characterized most of the plant homologues to the *Sar1*, *Sec12*, *Sec13* and *Sec23* [265,267,271,272]. Although COPII coat protein homologues have been identified in plants [226,267], COPII-coated vesicles have not been isolated or identified in situ. In vitro reconstitution experiments suggest that same protein machinery (Sar1, Sec12, Sec23/24, Sec13/31) is being used in plant cells for anterograde transport from ER to Golgi, but in some instances it is being used in different ways to serve specific plant needs (e.g. from ER-to-vacuole, etc.) [45,49].

21. Conclusion

Movement of proteins in the early secretory pathway is facilitated by two sets of coated vesicles: COPI vesicles carry cargo between Golgi cisternae and from the Golgi to the ER, whereas COPII vesicles carry newly synthesized proteins from the ER to Golgi. The coats of both types of vesicles are formed by the interaction of a cytosolic protein complex with a GTP-binding protein at the surface of donor membrane. At the core of the COPI and COPII budding machinery lie the small molecular weight GTPases Arf1 and Sar1. These GTPases act as binary switches for coat formation such that the GTP-bound state is active and the GDP-bound state is inactive. Arf1 is probably a major regulator of vesicle biogenesis in intracellular traffic, which not only controls assembly of both COPI- and AP1/clathrin-coated vesicles but also recruits other proteins to membranes, including some that may be components of further coats. The specific binding of Arf1-GDP to the Golgi membrane through the cytoplasmic domain of p23 cargo receptors has been shown to be the first step of Arf1 recruitment to the membrane and probably works as ARF receptor in the Golgi membrane. Recent structural and biochemical studies have provided a wealth of details of the interactions between Arf1/Sar1 and the proteins that regulate their activities as well as providing clues to the types of effector molecules that are controlled by Arf1 and Sar1.

Although most of the discoveries in vesicular trafficking field have resulted from studies of yeast and mammalian cells, plant researches are currently gaining enough ground in identifying the machinery involved in vesicular trafficking. These studies indicate that plant cells essentially use the same machinery for vesicular transport as mammalian or yeast cells but they have adapted several of these proteins to the unique requirements for transport between membrane compartments not found in other organisms.

Note added in proof

As we previously demonstrated with mammalian cells [143], Arf1 is first recruited in GDP form by cytosolic tails of p23 membrane proteins. Recently Contreras et al. [273] have also shown similar interaction with p24 membrane proteins in plant cells. Additionally they have also shown the cooperativity of both dilysine and phenylalanine motifs of these proteins in recruiting plant coatomers to the membranes.

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