Mechanisms of vesicle formation: insights from the COP system

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The major cytosolic and membrane proteins that represent machinery of coat protein (COP)-coated transport vesicles within the secretory pathway are characterized to date. This has allowed investigation of the molecular mechanisms that underlie the formation of these vesicles. *In vitro* binding studies and reconstitution experiments have provided insights at the molecular level into the biogenesis of COPII- and COPI-coated vesicles.

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Abbreviations

ARF	ADP-ribosylation factor
COP	coat protein
ER	endoplasmic reticulum
GAP	GTPase activating protein
SNARE	soluble N-ethylmaleimide sensitive factor attachment protein receptor
TGN	*trans*-Golgi network

Introduction

Intracellular protein transport in eukaryotic cells is mediated by small transport vesicles that are defined by their coat proteins: COPII-coated vesicles allow exit from the endoplasmic reticulum (ER), COPI vesicles carry proteins within the early secretory pathway (i.e. the ER and Golgi apparatus) and clathrin-coated vesicles mediate transport from the *trans*-Golgi network (TGN) and endocytic transport from the plasma membrane [1–3]. Additional types of coated vesicles might be characterized in the future.

Vesicular transport intermediates do not only perform delivery of cargo to various destinations: at the same time they regulate the steady state of the endomembrane system of an eukaryotic cell. In this review, we focus on the composition and the assembly of machinery needed to form COP-coated vesicles and discuss mechanisms that allow uptake of cargo. We provide a model for COPI vesicle formation, features of which could be generalized for other types of coated intermediates.

A general principle for coated vesicle formation

Assembly of the vesicle coat on the surface of a donor membrane seems to follow a general mechanism: it is initiated by the recruitment of a small GTPase in its GTP-bound state. Subsequent binding of hetero-oligomeric protein complexes to the membrane induces deformation of the membrane and appearance of a coated bud. Membrane proteins of the donor organelle play an important role during coat assembly. Transmembrane proteins might serve two functions at the same time: one on the cytosolic side as coat receptors in the recruitment of soluble coat protomers, and another role in the lumen in the sorting of cargo proteins. Thus, coat assembly is likely to link vesicle formation with sorting of cargo [4].

In vitro reconstitution of coated vesicle formation from biological membranes

COPII-coated vesicles were reconstituted *in vitro* from washed microsomes and three purified soluble protein

Table 1

Coat proteins of COPII- and COPI-coated vesicles.

	Subunits				
Protein complex	Mammals	Yeast	Size	Features	Interactions
COPII					
Sec13 complex	hSec13p	Sec13p	~34 kDa	WD-40 repeats	
	hSec31p	Sec31p	~150 kDa	WD-40 repeats	
Sec23 complex	hSec23p	Sec23p	~85 kDa	GAP for Sar1p	Bos1p, Bet1p
	hSec24p	Sec24p	~105 kDa		
Sar1	hSar1p	Sar1p	~21 kDa	Ras family of GTPases	Bos1p
COPI					
Coatomer	α-COP	Ret1p	~140 kDa	WD-40 repeats	β′-COP, ε-COP
	β-COP	Sec26p	~107 kDa		δ-COP, ARF1
	β′-COP	Sec27p	~102 kDa	WD-40 repeats	α-COP
	γ-COP	Sec21p	~97 kDa		ζ-COP, ARF1, KKXX motifs, p24 family
	δ-COP	Ret2p	~57 kDa		β-COP
	ε-COP	Sec28p	~35 kDa		α-COP
	ζ-COP	Ret3p	~20 kDa		γ-COP
ARF 1	ARF 1	yARF 1/2/3	~20 kDa	Ras family of GTPases	β-COP, γ-COP

factors: Sar1p, Sec13p complex and Sec23p complex [5] (Table 1). COPI-coated vesicles have been assembled stepwise from isolated Golgi membranes and the cytosolic components ADP-ribosylation factor 1 (ARF1) and coatomer (a heptameric protein complex) [6] (Table 1). In both cases, the initial step in coated vesicle formation involves recruitment of a small GTPase to the target membrane — Sar1p·GTP in the COPII system and ARF1·GTP in the COPI system — a reaction catalyzed by guanine nucleotide exchange factors. The integral membrane protein Sec12p [7] mediates nucleotide exchange on Sar1p, whereas the exchange reaction on ARF1 is catalyzed by factors that are recruited to the membrane from the cytosol [8]. Activation of the small GTPases precedes recruitment of the remaining coat subunits to the membrane. Formation of the COPII coat occurs in two steps: first, the Sec23p complex is bound followed by recruitment of the Sec13p complex. In contrast, COPI-coated bud formation is initiated by recruitment of preassembled coatomer (coat protomer) [9]. This involves a direct interaction with membrane-bound ARF1·GTP and coatomer via its β-COP and γ-COP [10,11] and results in clustering of ARF1·GTP and deformation of the membrane into a coated bud.

Fusion of vesicles with their target membrane requires prior dissociation of the coat. This is achieved by the activity of GTPase activating proteins (GAP) that increase the rate of hydrolysis of GTP bound to Sar1p or ARF1 and leads to dissociation of the G proteins from vesicles thus destabilizing the coat. In the COPII system, Sec23p (part of the coat complex) acts as a GAP for Sar1p [12], whereas in the COPI system an ARF specific GAP is recruited to the membrane from the cytosol [13]. GTP hydrolysis by ARFGAP was found to be increased by coatomer and this led to the suggestion of a tripartite complex of ARF, ARFGAP and coatomer at the membrane of a COPI vesicle [14••]. Upon dissociation of the coat, vesicles can fuse with the target membrane. In the presence of nonhydrolyzable analogues of GTP, the COP proteins are retained and thus vesicles accumulate.

Membrane proteins in vesicle formation and cargo selection

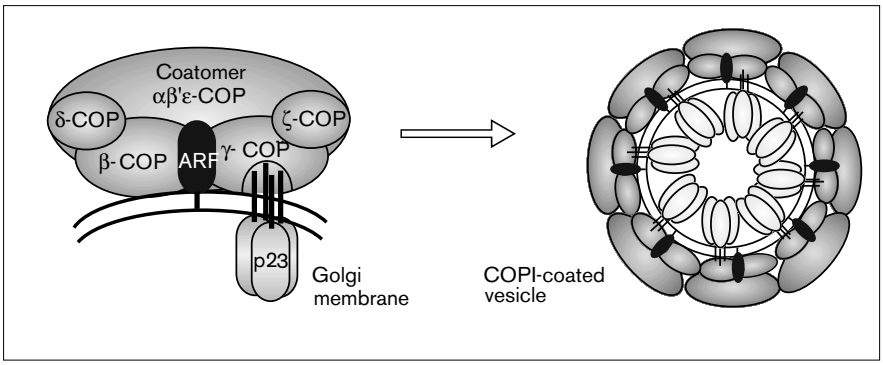
It is useful to make a conceptual difference between membrane proteins that serve as cargo and those proteins that constitute the machinery of COP-coated vesicles. Whereas cargo has to be delivered unidirectionally, machinery must be cycled. The cytosolic machinery has been outlined above, and membrane machinery comprises coat/cargo receptors and targeting molecules, the SNAREs.

An interaction between a cytoplasmic domain of a membrane cargo protein of the early secretory pathway and a coat component was originally reported for an ER-retrieval motif (KKXX) and coatomer [15] and was attributed subsequently to the γ-subunit of the complex [16]. This was a first indication that COPI-coated vesicles might be involved in retrograde-directed selective protein transport from the Golgi to the ER and initiated a still ongoing debate about the directions COPI vesicles might take. The issue of retrograde versus anterograde transport of COPI vesicles has been discussed in previous reviews [17,18].

Sorting of membrane cargo into a COPII prebudding complex has been described for several proteins in yeast [19••] and mammals [20,21••] and is believed to be mediated via an interaction with the Sec23p complex [21••], although no direct binding of these components has been demonstrated. Thus, sorting of membrane cargo could occur by a direct interaction between the cytoplasmic domain of the cargo protein and coat components. Sorting of soluble cargo requires involvement of transmembrane receptors, which might couple sorting in the lumen of an organelle to coat assembly at the cytoplasmic face. The expected properties of a transmembrane cargo receptor include one or more transmembrane domains, a lumenal domain able to interact with cargo species and a cytoplasmically exposed domain that interacts with coat subunits. Further, such proteins would be expected to cycle between ER and Golgi. To date, two types of protein are known that fulfill these criteria. One is typified by the KDEL receptor — a multispanning membrane-protein that recognizes a carboxy-terminal KDEL tetrapeptide (Lys–Asp–Glu–Leu) of lumenal soluble proteins of the ER and retrieves from the Golgi those KDEL proteins that have escaped from the ER [22]. Accordingly, the KDEL receptor has been used to localize putative retrograde-directed COPI-coated vesicles [23].

Another type of vesicular transmembrane protein is referred to as the p24 family. These are type I membrane proteins, some of which have been found in COPII- [24] and COPI-coated vesicles [25,26]. They share a common structural organization: a large lumenal domain with the propensity to form coiled-coils, and a short cytoplasmic domain that contains two conserved motifs: a diphenylalanine motif and, in most cases, a dibasic motif at their carboxyl termini. In yeast, two p24 family members, Emp24p [24] and Erv25p [27], have been localized to ER-derived COPII coated vesicles. These proteins form a complex that is required for efficient ER to Golgi transport of a subset of secretory proteins [24,27]. Homologues of Emp24p and Erv25p have also been identified in mammals and designated p24 [25,28] and p23 [26,28,29], respectively. These were the first transmembrane proteins to be identified in COPI-coated vesicles. As p24 and p23 are especially abundant in Golgi membranes and are concentrated into Golgi-derived COPI-coated vesicles, where they are present in approximately stoichiometric amounts relative to coatomer and ARF, these proteins are likely to be necessary for COPI-dependent budding. This is supported by the finding that the cytoplasmic domains of p23 and p24 are able to bind to coatomer [26,30] and are required for cycling of these proteins within the early secretory pathway [30,31].

Figure 1



Model for COPI-coated vesicle formation. A trimeric complex of coatomer with membrane-bound ARF and a tetramer of cytoplasmic tails of p24 members, for example p23, is suggested to provide the molecular basis for budding. In this trimeric complex direct interactions exist between ARF and the β- and γ-subunits of coatomer, and between p24 cytoplasmic domains and γ-COP. Figure reproduced with permission from [54].

More recently, it was shown that, in addition to their COPI binding, p23 and p24 are also able to bind to COPII *in vitro* and to form hetero-oligomeric complexes with various other p24 family members (p25, p26, and p27) [32,33]. COPII binding was suggested to involve their diphenylalanine motif [32], whereas interaction with COPI depends on both their diphenylalanine and dibasic motifs [26,32]. On the basis of these observations it is tempting to hypothesize that the state of oligomerization of p24 complexes might regulate their interaction with a certain type of coat and thus define whether p24 proteins are active in COPII or COPI vesicles [33].

The above data have made p24 proteins strong candidates to act as coat receptors. In contrast, their putative role as cargo receptors is less clear. Although in yeast several lines of evidence support a role for them in selective sorting [19••,27], a direct interaction with cargo molecules remains to be demonstrated.

Another class of membrane proteins that is present in COPII- [34] and also COPI-coated vesicles [35] is the v-SNAREs (vesicle soluble N-ethylmaleimide sensitive factor attachment protein receptors) [36] — part of the targeting and fusion machinery of the secretory pathway [37,38]. v-SNAREs can interact with COPII components (and possibly with COPI, although this has not been demonstrated) in order to become enriched in departing vesicles [39••]. An aggregate that includes the v-SNARE Sec22p and COPII components has been described recently [19••]. Furthermore, an interaction has been shown between the v-SNAREs Bet1p and Bos1p and the Sec23p complex in the presence of Sar1p·GTP [39••]. Concentration of Sec22p in reconstituted COPII liposomes was previously reported in a liposome budding experiment [40••]. Similarly, complexes between v-SNAREs and components of the COPI coat might exist.

Reconstitution of coated vesicles from chemically defined liposomes

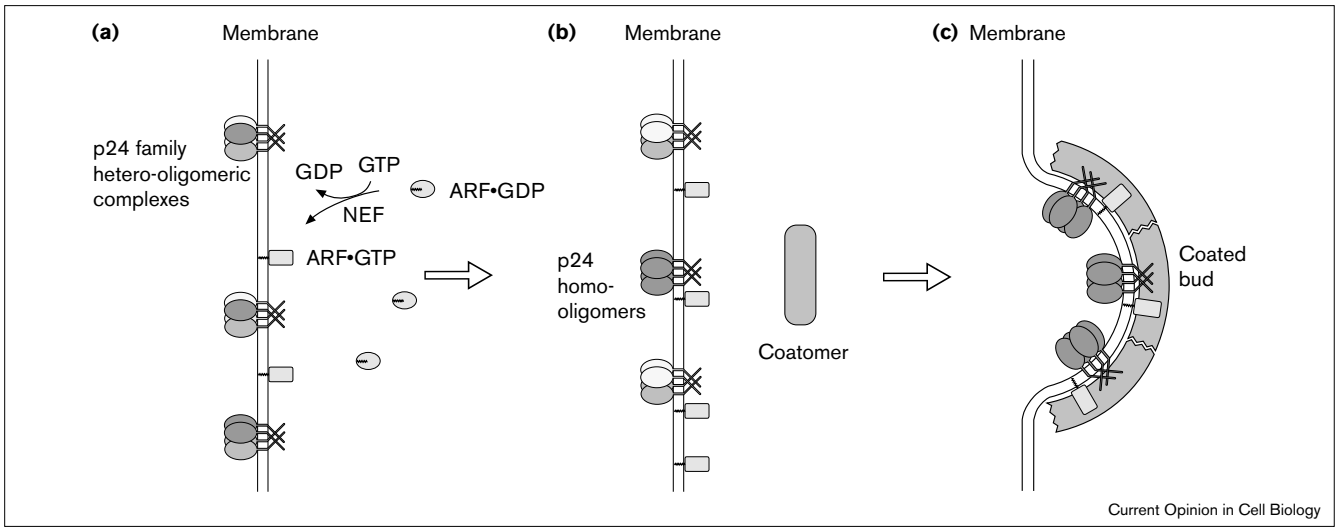
In order to define the minimal requirements for the formation of COP-coated vesicles, their budding was reconstituted from chemically defined liposomes. Using this reductionist system, it was shown that all that is needed to form a COPI-coated vesicle are the cytosolic proteins ARF and coatomer and the cytoplasmic domains of coat/cargo receptors (p24 family) emanating from the bilayer surface [41••]. Liposome-derived budding requires the presence of GTP and an elevated temperature of 37°C similar to the observed requirements for COPI vesicle formation from biological membranes. This budding reaction was shown to be completely independent of the lipid composition of donor liposomes provided that the cytoplasmic tail of p23 (or another p24 family member) was present. As only the cytoplasmic domains of the p24 proteins are essential in this reconstitution, an interaction of p24 lumenal domains with cargo proteins does not seem to be necessary for coat assembly, which is consistent with early observations [42]. COPI- (and COPII-) coated vesicle formation from chemically defined liposomes without the need for any membrane protein was reported depending on the presence of high amounts of acidic and unsaturated phospholipids [43,44•]; however, this budding reaction was independent of temperature and required a nonphysiological composition of phospholipids as well as the presence of a nonhydrolyzable analogue of GTP. Thus, this reconstitution is likely to represent a partial event in budding, revealing a role of the coat proteins in shaping the membrane.

Taken together, reconstitution of COPI vesicle budding from artificial lipid bilayers together with the identification of binding sites both for ARF1·GTP at the β-COP and γ-COP and for p23 within γ-COP provide the molecular basis of COPI coat assembly: a bivalent interaction of coatomer to form a trimeric complex between the membrane-bound ARF1·GTP and the cytoplasmic tails of p24 family members (Figure 1).

Polymerization of coatomer and COPI bud formation

With the knowledge of the minimal requirements for COPI budding, a question still remains. How does recruitment of a coat drive formation of a curvature in a membrane? An answer to this basic question might come

Figure 2



Hypothetical model for a putative role of ARF1•GTP in the assembly of a COPI-coated vesicle and uptake of cargo. **(a)** ARF1•GTP (rectangle) is targeted to the membrane upon nucleotide exchange catalyzed by an exchange factor (NEF). **(b)** Membrane-bound ARF1•GTP might be involved in the dissociation of hetero-oligomeric complexes of p24 proteins. This might result in the formation of homo-oligomers, which represent high affinity binding sites for coatomer at the cytoplasmic face and are able to interact with selective cargo (not shown) on the lumenal side. Sorting of cargo requires hydrolysis of ARF-bound GTP. **(c)** Coatomer interaction with p24 homo-oligomers induces a conformational change and polymerization of the complex, which shapes the membrane into a coated bud.

from *in vitro* binding studies with coatomer and the cytoplasmic tail peptide of p23 [45••]. The results of these studies suggest that a tetramer of p23 induces specific polymerization of the coatomer complex. Polymerization of coatomer is accompanied by a conformational change in the complex resulting in an increased susceptibility of its γ-subunit to protease (the direct binding partner of p24 family cytoplasmic domains [46•]). Strikingly, this conformational change and polymerization of coatomer is specifically induced by some p24 cytoplasmic domains but not by a peptide with a characteristic KKXX ER-retrieval motif that binds coatomer with similar efficiency and via the same subunit as p23 [46•]. Thus, the two classes of cytoplasmic domains of coatomer binding proteins seem to have two distinct and different functions: interaction of a retrieval motif might serve the sorting of membrane cargo into retrograde COPI vesicles. The p23 and p24 proteins, however, represent part of the machinery of a COPI vesicle.

Hydrolysis of GTP and uptake of cargo

COPI vesicles were generated *in vitro* from Golgi membranes that contained comparable amounts of retrograde and anterograde cargo, and their cargo contents analyzed. Surprisingly, both types of cargo were included into these vesicles in the presence of GTP in similar large amounts and in only small amounts in the presence of the slowly hydrolyzable GTP analogue, GTPγS [47•]. A similar observation was made in an *in vivo* system (R Pepperkok, JA Whitney, M Gomez, TE Kreis, personal communication). Thus, hydrolysis of GTP seems to be needed for the assembly of a complete COPI vesicle. A likely candidate for the corresponding GTPase activity is ARF1 (R Pepperkok, JA Whitney, M Gomez, TE Kreis, personal communication). Interestingly, coatomer cannot bind to Golgi membranes in the absence of ARF and GTP (or GTPγS) [48], although the coatomer receptor proteins (e.g. p23 or p24) are present in the Golgi. On the other hand, coatomer is able to bind to the cytoplasmic tails of these type I membrane proteins, either in solution or coupled to sepharose beads [26,45••]. To explain these observations, one must assume that the cytoplasmic domains of p24 proteins in the nonprimed Golgi are not available for interaction with coatomer. In fact, a complex containing a variety of these proteins has been described that resists solubilization by mild detergent [32]. Thus, in a hetero-oligomeric state, p24 family proteins might not be available for coatomer binding and one role of ARF·GTP might be to dissociate coatomer receptors from this complex. Individual coatomer receptors might then form homo-oligomers, most probably their high affinity state for coatomer binding [45••] (Figure 2). If p24 family proteins can act as cargo receptors, some time must be allowed not only for their dissociation but also for lateral diffusion of cargo into the budding site, where it can interact with the lumenal domains of p24 proteins. This time-window might be kept open by several cycles of ARF·GTP hydrolysis. In ARF·GTPγS, however, the nucleotide cannot be hydrolyzed and therefore ARF stays fixed after its first encounter with the membrane and would release a stoichiometric amount of p24 proteins making them available for coatomer binding and not allow for the time needed for diffusion of cargo into the

coating zone. This explanation for the observation that GTP hydrolysis is necessary for cargo uptake is speculative at this time, however, it is consistent with circumstantial evidence.

ARF-GTPase activity linked to coatomer-mediated uncoating?

From recent work by Goldberg [14••] we have learned that ARF1·GTP can exist in three different states: ARF1·GTP with virtually no GTPase activity; ARF1·GTP complexed with an ARFGAP, with GTPase activity of about 1/1000 of the corresponding RasGAP complex; and a ternary complex of ARF1, ARFGAP and coatomer, with a GTPase activity about 1000-fold higher than the ARF1–ARFGAP complex.

This fits perfectly with the finding that in the ARF1–ARFGAP complex the effector site is open and therefore accessible for coatomer as an effector. This is in contrast to the RasGAP complex, where the effector site is covered by the GAP protein [49], and in agreement with the position of amino acid residues in ARF1 shown to interact with coatomer [10,11]. Goldberg has suggested that in the ternary complex the active center for GTPase activity is perfectly aligned and that it is coatomer which provides the structural element, a so-called Arginine finger, known to activate GTPase activity of small G proteins. As at least two ARFGAPs seem to exist in a given eukaryotic cell [13,50–53]. It is extremely tempting to speculate that the lower GTPase activity of ARF in the bimodal complex with a GAP could serve to keep the time-window open needed for uptake of cargo to form a complete COPI vesicle (see above) and that this GAP is lost once the coat has formed. A second GAP, somehow specifically located at the target membrane, would then enter the ARF1–coatomer complex on the surface of the vesicle once it has docked to the target membrane and stimulate GTPase activity by a factor of 1000, which is high enough to result in efficient uncoating. This is also consistent with Goldberg's finding that an ARF1·GTP coatomer interaction alone does not stimulate GTPase activity at all. This hypothesis can be tested by assaying the different GAPs *in vitro* for their affinities to ARF and ARF–coatomer and by determining their degree of GTPase activation as well as their intracellular localization.

Conclusions

The general mechanisms underlying the formation of a transport vesicle are understood at a molecular level. In order to understand the budding reaction in more detail, several future challenges must be overcome: these will include assessment of the roles of the various p24 family members (this might well be linked to the elucidation of the directions COPI vesicles take); investigation of a possible sorting of lipids into transport vesicles; the establishment or dismissal of a role for p24 family members as cargo receptors; investigation of whether a conformational change of coat proteins induced by binding to their receptors is a general principle in coat polymerization, which thus shapes the membrane into a bud; and understanding the need for GTP hydrolysis for the formation of a complete COP-vesicle and for its uncoating at a molecular level.

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