Minireview

Biogenesis of transport intermediates in the endocytic pathway

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Abstract Evidence is accumulating that membrane traffic between organelles can be achieved by different types of intermediates. Small (< 100 nm) and short-lived vesicles mediate transport from the plasma membrane or the trans-Golgi network to endosomes, and formation of these vesicles depends on specific adapter complexes. In contrast, transport from early to late endosomes is achieved by relatively large (~0.5 μm), long-lived and multivesicular intermediates, and their biogenesis depends on endosomal COP-I proteins. Here, we review recent work on the formation of these different transport intermediates, and we discuss, in particular, coat proteins, sorting signals contained in cargo molecules and the emerging role of lipid in vesicle biogenesis.

Key words: Adapter; COP protein; Sorting; Endosome; Multivesicular body

1. Introduction

The endocytic pathway is responsible for the internalization, recycling and degradation of extracellular material, and the turnover of cell surface components [1,2]. Internalized solutes, lipids and proteins first appear in early endosome, and then recycle back to the plasma membrane for reutilization, or are targeted to late endosomes and eventually lysosomes for degradation (see outline Fig. 1). Protein trafficking from the cell surface to lysosomes involves distinct steps of protein sorting and occurs via different types of vesicular intermediates.

Selective protein transport from plasma membrane to early endosomes is mediated by small (< 100 nm diameter) and short-lived (~1 min) vesicles coated with clathrin and the AP2 adapter complex [3]. The majority of internalized molecules are recycled back to the cell surface, whereas a small fraction, including down-regulated cell surface receptors, is selectively incorporated within transport intermediates destined for late endosomes [1,2]. These intermediates, which will be referred to here as endosomal carrier vesicles (ECVs) [4–6], are relatively large (~0.5 μm) and long-lived (~15–30 min) vesicles with a multivesicular appearance, in contrast to AP2/clathrin-coated vesicles. ECV biogenesis depends on proteins which belong to the COP-I coat, previously known to be involved in the early secretory pathway [7]. Protein transfer from late endosomes to lysosomes may occur via a different mechanism involving the transient formation of a hybrid organelle [8,9].

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Endosomes are also connected to the biosynthetic pathway via selective transport routes. Vesicles coated with clathrin associated with the AP1 adapter complex mediate delivery of newly synthesized lysosomal enzymes and lysosomal proteins from the trans-Golgi network (TGN) to endosomes, as well as perhaps AP3-coated vesicles [10,11]. Protein recycling back to the TGN occurs from late endosomes [12], as well as perhaps from early endosomes [13]. The different types of transport intermediates and coat proteins involved during transport toward lysosomes from the cell surface or from the TGN will be discussed further in this review.

2. Clathrin-coated vesicles at the plasma membrane

At the cell surface, molecules destined to be internalized cluster within plasma membrane invaginations coated with clathrin and the AP2 adapter (coated pits). These invaginations pinch off from the membrane, become free vesicles, and fuse with early endosomes, thereby delivering the internalized cargo of solutes, proteins and lipids. It is generally accepted that the coat is responsible for the sorting of internalized proteins, and that its polymerization contributes to the invagination process. The AP2 adapter is a heterotetrameric complex, containing two ~100 kDa proteins (α, β2 adaptins), a ~50 kDa chain (μ2) and a small chain of ~20 kDa (σ2) [3].

AP2 can interact directly with Tyr-based or di-Leu motifs present in the cytoplasmic domains of cell surface receptors, thereby allowing their selective incorporation within forming coated pits [14]. Recruitment of AP2 on the plasma membrane may depend, at least in part, on interactions with cell surface receptors via these sorting signals. Expression of the trans-

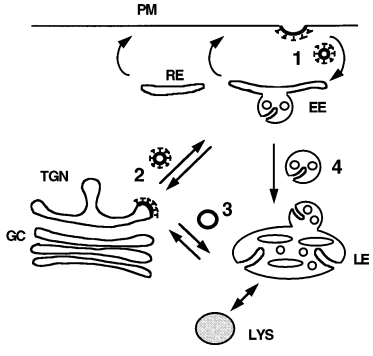

Fig. 1. Transport intermediates in the endocytic pathway. Major routes of membrane transport are outlined, and transport intermediates are indicated. 1: Clathrin/AP2-coatedvesicles; 2: clathrin/AP1-coated- esicles; 3: AP3-coated vesicles, presumably involved in transport from the TGN to endosomes/lysosomes; 4: endosomal carrier vesicles. Abbreviations: pm, plasma membrane; EE, early endosome; RE, recycling endosome; LE, late endosome; LYS, lysosome; TGN; trans-Golgi network; GC, Golgi complex.

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ferrin (Tf) receptor, which contains a Tyr-based internalization signal, promotes formation of a clathrin lattice, even though invaginated pits do not form [15]. Overexpression of chimeric proteins containing cytoplasmic domains with classical internalization signals to saturation level does not induce coated pit formation [16]. However, overexpression of HIV-1 viral Nef protein causes the assembly of clathrin-coated pits, but specific for CD4/Nef [17]. In contrast to membrane binding of AP1, AP3 and COP-I, AP2 assembly at the plasma membrane is not stimulated by GTPγS and does not depend on ARF1 [18,19]. In the presence of GTPγS, however, AP2 is recruited by endosomes in a process mediated by ARF1 and phospholipase D [20,21].

The precise mechanisms responsible for specific assembly of AP2/clathrin at the plasma membrane are still not clear, but new insights are expected from recent studies, which have uncovered new components of the clathrin/AP2 coat. A substrate of the EGF receptor tyrosine kinase, Eps15, was recently found to be constitutively associated with coated pits and is required for EGF and Tf internalization [22–26]. Epsin, which binds the EH domain of Eps15, may be another new component of the clathrin machinery, since epsin disruption blocks clathrin-mediated endocytosis [27]. After AP2 binding, clathrin molecules are recruited through interactions between the ear domain of AP2 and the clathrin triskelion legs, driving clathrin assembly [28], as confirmed by the structure of the clathrin coat at 21 Å resolution [29]. Once the clathrin/AP2 coat is assembled, the GTPase dynamin is required for fission of the vesicle from the plasma membrane [30]. GDP/GTP exchange leads to the self-assembly of the dynamin polymers which are thought to form a collar around the neck of the coated pit, GTP hydrolysis then providing the driving force for neck constriction and vesicle fission.

3. Clathrin-coated vesicles at the TGN

Clathrin-coated vesicles also form on the TGN, and are responsible for the sorting and transport of newly synthesized lysosomal hydrolases bound to the mannose 6-phosphate receptors (MPRs) towards endosomes [11,12]. These vesicles have the same morphological appearance as those present at the plasma membrane, but differ in composition, the clathrin lattice being associated with the AP1 adapter complex (γ, β1, μ1 and σ1). Unlike AP2, the binding of AP1 to TGN is mediated by ARF1, blocked by brefeldin A and enhanced by GTPγS [31].

AP1 recruitment onto TGN membranes was proposed to depend on MPRs, well characterized cargo molecules of TGN-derived clathrin/AP1-coated vesicles. Knock-out of MPRs in mice decreases AP1 binding onto TGN membranes [32], and binding is restored upon MPR re-expression [33]. However, it is not clear at present whether high affinity sites for AP1 binding are provided by MPR itself [32], ARF1 [34], or both. Recent studies also suggest that MPRs are not essential determinants in the initial AP1 binding step, but may play a regulatory role in clathrin-coated vesicle formation by affecting ARF·GTP hydrolysis [35]. Further work will be required to characterize the precise mechanisms responsible for AP1 localization to TGN membranes, much like for AP2 at the plasma membrane.

Clathrin assembly after AP1 binding is believed to occur in a process similar to clathrin/AP2 assembly. Fission of AP1/clathrin-coated vesicles may also depend on dynamin, as for AP2/clathrin-coated vesicles. Dynamin was found associated with the TGN [36,37], and antibodies against dynamin or dynamin-depleted cytosol inhibited clathrin/AP1 vesicle formation in vitro [38].

4. AP3

Recently, a new adapter-related protein complex, AP3, has been identified, both in mammals and in yeast [39–42]. Similar to AP1 and AP2, the AP3 adapter is a heterotetramer (δ, β3, μ3 and σ3). Studies in yeast showed that AP3 is involved in direct transport of alkaline phosphatase and Vam3p from a post-Golgi compartment to the vacuole [42–44]. However, the precise function of AP3 in higher eukaryotic cells is not clear yet. A homologue of AP3 δ adaptin has been found in *Drosophila* [45,46]. In mouse the *mocha* gene encodes δ AP3 adaptin and the *pearl* gene is probably the β3 adaptin homologue [47]. Disruption of these genes results in pigmentation defects and/or heavy bleeding, presumably due to defective biogenesis of lysosome-related organelles, such as melanosomes and platelet dense granules.

Much like AP1, but unlike AP2, AP3 membrane binding is sensitive to brefeldin A and enhanced by GTPγS, and requires ARF1 in vitro [48]. Whether AP3 is associated with clathrin is still a matter of debate [39–41]. Membrane proteins destined for late endosomes-lysosomes which can interact with the AP3 adapter have been identified. Although earlier studies suggested that Lamp1 may be incorporated into AP1-coated vesicles [49], it was recently shown that Lamp1 and LIMP-II overexpression promoted membrane binding of AP3, and inactivation of AP3 caused Lamp1 and LIMP-II mistargeting [50]. Consistent with these observations, mistargeting of the lysosomal proteins CD63, Lamp1, and Lamp2 was observed in fibroblasts from patients with Hermansky-Pudlak syndrome who carry mutations in the β3A subunit of AP3 [51]. AP3 was also shown to interact with the cytoplasmic tails of the LIMP-II and melanosome-associated protein tyrosinase in vitro, and the binding was dependent on a di-Leu sequence in both proteins [52]. These data collectively suggest that AP3 in mammalian cells is likely to be involved between TGN and lysosomes, similarly to the Golgi-vacuole pathway in yeast. However, AP3 has also been implicated in synaptic vesicle formation from endosomes [53], suggesting that AP3 may be required at more than one transport step.

5. ECVs and COP-I

ECVs exhibit a characteristic multivesicular appearance, and accumulation of internal membranes appears to be coupled to the organization/dynamics of early endosomes [54,55]. Once formed, ECVs are transported towards late endosomes in a process which requires intact microtubules and motor proteins [5,6], in contrast to clathrin-coated vesicles. Then, they fuse with late endosomes [5,6], and docking/fusion depends on NSF, αSNAP, as well as perhaps another AAA ATPase [56] and the small GTPase rab7 [57].

Protein motifs which function as endosome/lysosome-targeting signals have been identified, but it is often not clear whether these signals operate within endosomes, or at earlier transport steps [1,2,14]. However, an eight amino acids motif in the interleukin-2 receptor β chain was shown to determine

its transport from early to late endosomes (and lysosomes) [58,59]. Recent studies also revealed that HIV Nef contains two acidic amino acids critical for CD4 down-regulation, which operate within early endosomes and mediate specific interactions with endosomal βCOP [60]. Acidic clusters have previously been reported to function as intracellular sorting or localization signals [61–64].

In vivo and in vitro studies have shown that endosomal COP-I coat proteins are involved in ECV biogenesis and play a role in protein sorting [55,60,65–67]. The COP-I subunits are present as a protein complex termed the coatomer, including in the cytosol [7,68]. The coatomer is formed by seven polypeptides, α, β, β′, γ, δ, ε and ζCOP, and is known to be also involved at early steps of the biosynthetic pathway [7]. Binding of COP-I to biosynthetic [69] and endocytic (F. Gu and J. Gruenberg, unpublished) membranes is regulated by the small GTP binding protein ARF1. However, endosomal and biosynthetic COPs exhibit different properties.

Endosomal and biosynthetic COPs appear to differ in composition, since γ and δCOP subunits are not detected on endosomes, in contrast to biosynthetic membranes [65,66]. After εCOP degradation in the ldlF cell line, which carries a temperature-sensitive mutation in the εCOP gene [70], αCOP no longer binds endosomes but is still recruited onto biosynthetic membranes [55], consistent with observations that αCOP stabilizes εCOP in yeast [71]. Endosomal and biosynthetic COPs also differ in their biochemical properties. Neutralization of the endosomal pH inhibits ECV biogenesis in vitro [55,66], transport from early to late endosomes in vivo and in vitro [54,72], as well as transport to lysosomes [73]. Then, COP binding to endosomal, but not biosynthetic, membranes is inhibited [55,66], and ARF1 appears to regulate this process (F. Gu and J. Gruenberg, unpublished). COP assembly onto endosomes may thus be regulated by a trans-membrane pH sensor, thereby signaling the onset of the degradation pathway on early endosomal membranes [66]. Recent studies indicate that a pH-dependent mechanism is also involved during retrograde transport in the early secretory pathway [74], but this process does not appear to depend on a pH-sensitive COP binding step [55,66,74].

At present little is known about the direct role of endosomal COPs in protein sorting. Inactivation of COPs in the ldlF cells at the restrictive temperature [70] affects Tf receptor recycling to the cell surface [67], but not bulk recycling [55]. Similarly, neutralization of the endosomal pH retards Tf receptor recycling more than bulk membrane recycling. Endosomal COPs may play a role in Tf receptor sorting, but these effects may also result from early endosome disorganization after COP inactivation or pH neutralization [54,55]. More directly, COPs were recently shown to play a role in the sorting of down-regulated HIV-Nef/CD4 complex in early endosomes [60].

A working model for the membrane association of endosomal COPs is outlined in Fig. 2. Acidification causes a conformational change in the putative pH sensor, which then allows ARF1-GTP recruitment onto early endosome, and then endosomal COP-I are recruited by ARF1-GTP. A functional dissection of endosomal COPs using cytosol from ldlF cells incubated at the restrictive temperature indicates that the β, β′ and ζ subunits can become membrane-associated in the absence of α and ε (and γ and δ, which are normally not detected on endosomes) [55]. It is unlikely that endosomal COPs bind membranes as a partial coatomer complex (lacking the γ and δ subunits), since coatomer subcomplexes have not been found [75]. Alternatively, the complete coatomer may be recruited as a single unit, followed by rapid dissociation of γ and δ subunits, uncovering sites for specific interactions with cargo molecules, or functional equivalents of the γ and/or δ subunits associated with endosomal COPs may remain to be discovered. Finally, endosomal COPs may interact with Nef and/or other cargo molecules destined to be incorporated into forming ECVs.

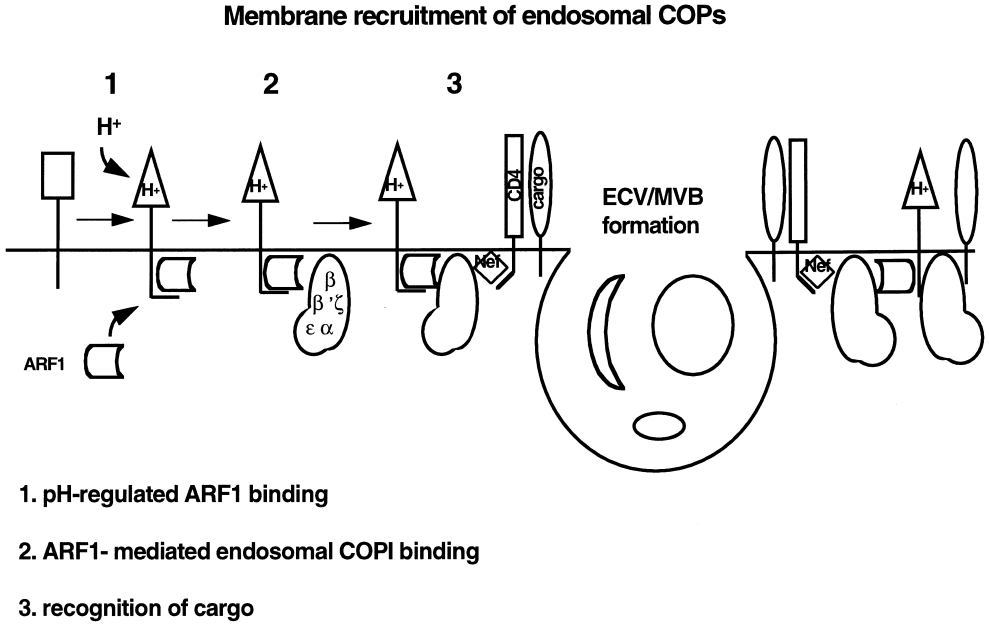

Fig. 2. Membrane recruitment of endosomal COPs. This model represents three steps presumably involved in endosomal COP membrane recruitment. 1: A transmembrane pH sensor regulates the binding of ARF1 onto the membrane. 2: ARF1 drives the binding of a specific set of endosomal COPs (α, β, β′, ε, ζ); the δ and γ subunits are not detected on endosomes). 3: Endosomal COPs recognize specific sorting motifs on cargo molecules as shown for HIV Nef/CD4, and mediate protein sorting and ECV formation.

6. Lipids and vesicle formation

Lipid-protein interactions in the endocytic pathway will be briefly mentioned here, since the possible roles of lipids and lipid metabolism in membrane traffic have been reviewed recently [76–79]. The neuronal protein synaptojanin, involved in synaptic vesicle recycling, is a $PI(3,4,5)P_3$ 5-phosphatase [80], and α adaptin (AP2) interacts with $PI(3,4,5)P_3$ [81]. Interactions of μ2 (AP2) with Tyr-based signal are increased by phosphoinositides phosphorylated in the D-3 position [82]. In contrast, interactions between the di-Leu motif and β1 (AP1) are inhibited by $PI(3,4)P_2$ or $PI(3,4,5)P_3$ [83]. Dynamin interacts with $PI(4,5)P_2$, thereby stimulating GTPase activity and promoting self-assembly [84,85]. Clathrin can polymerize onto liposomes, as does dynamin-1 with GTP hydrolysis then driving vesiculation [86,87]. Although it is too early to draw general models on lipid involvement in vesicle formation, these observations show that lipids, in particular signaling lipids, are regulators of various proteins involved in vesicle formation and molecular sorting.

It is not clear at present whether signaling lipids are involved in ECV biogenesis in mammalian cells. In yeast cells, however, phosphoinositides were reported to play a role in protein sorting in the multivesicular body [88]. In addition, COP-I recruitment onto Golgi membranes was proposed to depend on the local production of phosphatidic acid via ARF1-mediated phospholipase D activation [78], but the possible role of phospholipase D in ECV biogenesis is not known. Whereas little is known about signaling lipids, evidence is accumulating that internalized lipids do not distribute as bulk constituents of the bilayer, but that different lipids are selectively transported along recycling and degradation pathways, much like proteins [79]. Early and late endosomes also differ in lipid composition, and late endosomes contain high amounts of the unique lipid lyso-bisphosphatidic acid [89]. This lipid distributes exclusively within late endosome internal membranes, and is involved in protein sorting/trafficking within late endosomes [89]. Since lyso-bisphosphatidic acid is not detected in early endosomes, it is unlikely to be involved in ECV biogenesis. However, the fact that only some, but not all, internalized lipids are incorporated within forming ECVs suggests that these exhibit biophysical properties, including perhaps length and saturation of acyl chains [90], which contribute to the local deformation and subsequent invagination of membranes within forming ECVs.

7. Conclusions

Different types of intermediates ensure intracellular membrane flow along the endocytic pathway. Basic principles of protein sorting and membrane trafficking are shared by these intermediates (e.g. coat proteins), but differences exist in the molecular mechanisms (e.g. regulating coat association) (see Table 1). It is generally accepted that small, short-lived vesicles (i.e. clathrin-coated) provide the most efficient means to transport membrane constituents. ECVs, in contrast, are much larger, yet contain high amounts of internal membranes; it has not been possible to determine the surface area of internal membranes until now. Unlike AP-coated vesicles, ECVs, however, mediate microtubule-dependent transport over long intracellular distances, from peripheral early endosomes to perinuclear late endosomes [4–6], including from the tip of the axon the cell body in neurons [91]. ECV biogenesis may thus provide efficient membrane packaging in order to achieve optimal and cost-efficient long distance transport in the endosomal system.

The fact that COP inactivation leads to a disappearance of multivesicular domains from early endosomes [55] suggests that COPs directly participate in the process leading to the characteristic accumulation of internal membranes within forming ECVs. COP proteins may also be involved in membrane organization in the biosynthetic pathways, in addition to their known functions in protein sorting and vesicle formation, since COP inactivation in the ldlF mutant cell line at the restrictive temperature also leads to a disruption of Golgi organization [92]. The precise mechanisms leading to the invagination of internal membranes within forming ECVs are still not known. However, proteins and lipids are selectively incorporated within forming ECVs, in a process presumably facilitated by endosomal COPs. One may speculate that protein sorting and selective lipid partitioning within early endosomal membranes contribute to the association of some components within membrane regions or domains, which favor inward deformation of the membrane and then invagination, eventually leading to a forming ECV.

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Table 1
Membrane binding properties of coat proteins

	AP1	AP2	AP3	End COP	Golgi COP
ARF1	+ [31]	no (mis-loc) [21]	+ [48]	+[a]	+ [93]
PLD, PA	n.d.	no (mis-loc) [21]	n.d.	n.d.	+ [94]
Lumenal pH	n.d.	–	n.d.	− [55,66]	no [55,74]
Sorting motifs	diLeu, Tyr [95]	Tyr diLeu [95]	diLeu Tyr [52,96]	diGlu HIV-Nef [60]	KKXX, FF [68]
Subunits involved	μ1/Tyr [96]	μ2/Tyr [82]	μ3/Tyr [96]	βCOP/diGlu, Nef [60]	α,β,ε/KKXX [68]
		μ2/diLeu [99]			γ/KKXX [97]
	β1/diLeu [83]	β2/diLeu [100]			β,γ,ζ/FF [98]
PIs with D3 phosphate	−	+	n.d.	n.d.	n.d.
	β1/diLeu [83]	μ2 /Tyr [82]			

The table summarizes information on properties of coat proteins in the endocytic pathway. Greek letters refer to subunits of adapters and COPs (see text). Abbreviations: mis-loc: AP2 mis-localization to endosomes; +: stimulated; −: inhibited; no: no effect; n.d. not determined; diLeu: di-Leu-based sorting motif; Tyr: Tyr-based sorting motif; diGlu HIV-Nef: a two-Glu motif in HIV-Nef protein; KKXX: di-Lys ER; FF: di-Phe: sorting motifs in the early secretory pathway; End COP: endosomal COP-I; Golgi COP: Golgi COP-I; PLD: phospholipase D; PA: phosphatidic acid; PIs: phosphatidylinositides.
[a]F. Gu and J. Gruenberg, unpublished.

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