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Mechanisms of protein sorting and coat assembly: insights from the clathrin-coated vesicle pathway

Roland Le Borgne and Bernard Hoflack*

Clathrin-coated vesicles have provided the best example illustrating how both soluble and membrane proteins are selectively clustered into a transport intermediate for subsequent delivery to another intracellular compartment. Like cytosolic clathrin adaptors, the adaptor-like complex AP-3 binds to specific membranes and selects membrane proteins by interacting with their sorting signals.

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Abbreviations

ALP	alkaline phosphatase
AP	adaptor protein complex
ARF	ADP ribosylation factor
β-NAP	β-neuronal adaptin-like protein
CCV	clathrin-coated vesicle
COP1	coat protein or coatomer 1
MPR	mannose-6-phosphate receptor
TGN	*trans*-Golgi network

Introduction

In eukaryotic cells, clathrin-coated vesicles (CCVs) mediate the endocytosis of plasma membrane receptors and the sorting of transmembrane proteins in the *trans*-Golgi network (TGN) for transport to endosomes [1–3]. The first step in CCV formation is the translocation of cytosolic adaptor protein complexes (APs) onto their target membranes, a process regulated by an ADP ribosylation factor GTPase and phospholipids. APs concentrate transmembrane proteins by interacting with their tyrosine- and dileucine-based sorting signals, which are contained in their cytoplasmic domains [4]. They also interact with clathrin triskelions which, organized into cages, constitute the outer layer of the coat. Some of these fundamental aspects of coat assembly shared by the adaptor-like protein AP-3 will be discussed here.

Adaptor and adaptor-related complexes

The purification of CCVs led initially to the biochemical and molecular characterization of two related heterotetrameric adaptor complexes AP-1 and AP-2 (see Table 1). AP-1 is found associated with TGN-derived vesicles, AP-2 with those derived from the plasma membrane (reviewed in [5]). More recently, an adaptor-like complex referred to as AP-3 has been identified [6–8]. Like AP-1 and AP-2, AP-3 is a heterotetramer formed of two large δ (160 kDa) and β3A (120 kDa) subunits associated with the μ3 (47 kDa) and σ3 (22 kDa) subunits (Table 1). This adaptor-related complex is ubiquitously expressed, although variants of the β3 (β3B or the neuronal adaptin-like protein [β-NAP]) and μ3 (μ3B) subunits are specifically expressed in neuronal cells, in which AP-3 was first characterized [6–9].

The finding that the mammalian δ subunit was the homologue of the Garnet gene product in *Drosophila* gave the first clue to AP-3 function [10,11]. Mutations in this gene result in reduced pigmentation of melanosomes of the eyes and other tissues. The fact that melanosomes are lysosome-like organelles suggested that AP-3 may function in the biosynthetic transport of proteins to these organelles [9,11]. Yeast genetics gave strong support to this notion. Deletion of any of the AP-3 subunits in yeast results in a selective mislocalization of the vacuolar membrane protein alkaline phosphatase (ALP) and the vacuolar t-SNARE Vam3p without affecting carboxypeptidase Y sorting to the vacuole/lysosome [12••,13•,14]. In mammalian cells, the partial inactivation of AP-3 function with antisense oligonucleotides also leads to the selective misrouting of several lysosomal glycoproteins (Lamp-1 and Limp II) to the cell surface without affecting the AP-1-dependent transport of the mannose-6-phosphate receptors (MPRs) which transport lysosomal hydrolases from the TGN to endosomes [15]. This shows that sorting by AP-1 and AP-3 takes place independently.

Fourteen pigment dilution mutants in mice have also been described [16]. These pigment abnormalities are generally combined with a tendency to bleeding caused by abnormalities (platelet storage pool deficiency) in the endosome/lysosome-like organelles, platelet-dense granules [16]. While pigment abnormalities could result from a defect in any of several genes, one of these mutants (Pearl) exhibits defects of the β3A subunit of AP-3 [17]. Thus, in both yeast and mammalian cells, AP-3 functions in the lysosomal delivery of selected cargo proteins, a notion consistent with localization studies showing that, in mammalian cells, AP-3 is associated with the Golgi region, presumably the TGN [9,10], and with peripheral structures some of which contain endocytic tracers [8,18•].

β-NAP was initially identified, in the brain as a coat component associated with transport vesicles isolated from purified synaptosomes [6]. It has been recently shown that synaptic vesicles can be coated *in vitro* with AP-3, suggesting that this coat could be involved in synaptic vesicle biogenesis [19•]. Since synaptic vesicles can also be formed by a pathway involving AP-2, clathrin and dynamin [20], a possible explanation is that synaptic vesicles originate from two different locations, the plasma membrane (AP-2-dependent pathway) and endosomes (AP-3-dependent pathway).

Table 1

Adaptors and adaptor-related complexes*.

Adaptor	Subunits		Interactions†	Localization‡	Function
	Mammals	Yeast			
AP-1	γ	Apl4p	Unknown	TGN, early endosomes [38]	Biosynthetic intracellular transport to endosomes
	β1	Apl2p	Clathrin, dileucine-based motifs		
	μ1	Apm1p	Tyrosine-based motifs		
	σ1	Apa1p	Unknown		
AP-2	α_a,α_c		Unknown	Plasma membrane	Endocytosis
	β2		Clathrin, dileucine-based motifs?		
	μ2		Tyrosine-based motifs		
	σ2		Unknown		
AP-3	δ	Apl5p	Unknown	TGN?, endosomes [8–10,18•]	Biosynthetic intracellular transport to lysosomes/melanosomes Synaptic vesicle formation
	β3A/β3B (or β-NAP)	Apl6p	Clathrin?, dileucine-based motifs?		
	μ3A/μ3B	Apm3p	Tyrosine-based motifs		
	σ3A/σ3B	Aps3p	Unknown		

*This table summarizes the subunit composition of the mammalian assembly proteins. The yeast homologues are also listed. Four yeast gene products (Apl1p, Apl3p, Apm4p and Aps2p) may represent the homologues of AP-2 subunits. A clear function in transport, however, has only been demonstrated for the yeast AP-3 complex [12••]. †Here are listed only the interactions of adaptor protein complexes (APs) with clathrin and sorting signals. APs also interact with other components (see Schmid, McNiven and De Camilli, this issue, pp504–512). ‡In neuroendocrine cells, AP-1 is also found on immature secretory granules [40]. Under *in vitro* conditions AP-2 binds to late endocytic structures [53,54]. The dual localization of AP-3 is not understood. Presumably, the APs exhibit the same sorting function.

Thus, the present evidence suggests that, surprisingly, AP-3 is involved in two apparently unrelated events, the biogenesis of both lysosomes/melanosomes and synaptic vesicles. Mouse genetics may clarify this point. Mutations of the δ subunit, common to both the ubiquitous and brain-specific forms of AP-3, are expected to result in both pigment dilution and neurological disorders.

Clathrin binds AP-1 and AP-2 via the carboxy-terminal appendage domain of their β1 and β2 subunits [21]. Although early morphological and biochemical studies suggested that AP-3 was a component of non clathrin coats [9,10], the mammalian AP-3 can associate *in vitro* with clathrin by interaction of the β3 appendage domain with the amino-terminal domain of the clathrin heavy chain [18•]. This β3 appendage domain, conserved in β1 and β2 and other clathrin-interacting proteins, contains a motif for clathrin binding. Colocalization of AP-3 and clathrin on buds at the end of endosomal tubules and on the vacuolar part of endosomes is also observed in HeLa cells [18•]. In yeast, however, the AP-3-dependent transport of ALP is not affected in clathrin-deletion mutants [14]. Thus, whether or not protein sorting mediated by AP-3 depends on clathrin is still controversial.

Adaptor protein complexes interact with tyrosine- and dileucine-based signals

The transmembrane proteins that are endocytosed via the AP-2-dependent pathway or that follow the intracellular AP-1- or AP-3-dependent pathways to endosomes/lysosomes contain short amino acid sequences in their cytoplasmic domains that facilitate their sequestration into transport intermediates. These sorting determinants (reviewed in [4]) are known as tyrosine-based (motif Tyr-X-X-ϕ or Asn-Phe-X-Tyr, where X is any amino acid and ϕ is an amino acid with a bulky hydrophobic side chain) or dileucine-based sorting signals (two leucine residues or one leucine and a small hydrophobic residue). Each type of sorting signal can be found as a single copy, as illustrated by the short tails of lysosomal glycoproteins, or as multiple copies, or in combination, as illustrated by the MPRs.

In vitro binding assays have now shown that purified mammalian AP-1 and AP-2 can interact directly with tyrosine-based and dileucine-based sorting signals [22–25]. More recently, the AP-3 complex has also been found to interact with the dileucine-based motif of the cytoplasmic domains of Limp-II, a lysosomal glycoprotein, and of tyrosinase, a melanosomal protein [26]. An important aspect of research during the past few years has been to identify the AP subunits interacting with these sorting signals. In the yeast two-hybrid system, the μ chains of AP-1, AP-2 and AP-3 interact with tyrosine-based signals [23,27]. These interactions are mediated by the carboxy-terminal domain of μ1 and μ2 [28] and probably of μ3. Tyrosine-based and dileucine-based signals do not compete for each other during endocytosis, suggesting that these two types of sorting signals are decoded by two distinct entities [29]. The two-hybrid system has failed thus far to uncover interactions of AP subunits with dileucine-based sorting signals, but the cross-linking of short peptides to purified AP-1 has shown that its β1 subunit binds dileucine-based signals [30•], thereby leaving the possibility that the β2 and β3 homologues also bind

this sorting motif. Thus, these findings could explain how a tyrosine- and a dileucine-based sorting signal could both contribute to the efficient sorting of a single membrane protein bearing these two signals [31].

Our current understanding of the fine specificity of sorting, however, remains limited. Why can a plasma membrane receptor which undergoes AP-2-dependent endocytosis escape the AP-1-dependent lysosomal targeting during its biosynthetic transport, or why do Limp II and the MPRs (both of which contain a dileucine-based signal critical for endosomal/lysosomal targeting) interact with AP-1 and AP-3 in an opposite manner [15,26]? This could be explained by the relative affinity of APs for different signals. The amino acid composition of sorting signals, which are highly degenerate, could explain such differences [27,32]. Additional amino acid sequences, such as acidic clusters [33–37] may also modulate the accessibility of tyrosine- and dileucine-based sorting signals to APs. Post-translational modifications, such as phosphorylation [35,38–40], palmitoylation [41] or ubiquitination (reviewed in [42]), or other factors such as the oligomerization state of membrane proteins [43] can be potent determinants. Accessory proteins such as ABP280, an actin-binding protein which specifically decreases the endocytosis rate of furin [44], or the HIV-1 Nef protein, which associates with and increases the endocytosis rate of CD4, the major HIV-1 receptor [45,46•,47•], may modulate the interaction of specific membrane proteins with APs. Finally, the lipid composition of membranes, especially some phosphoinositides which regulate membrane traffic [48,49] could modulate the affinity of APs with sorting signals.

Recruitment of adaptor protein complexes to membranes

A major goal is to understand how APs recognize their target membranes, a question which has remained unanswered thus far. Binding of both AP-1 [50] and AP-3 [8,9,19•] is regulated by ADP ribosylation factor (ARF-1), a small GTPase whose binding to membranes is impaired in the presence of brefeldin A. ARF GTPases could function directly in coat assembly by interacting with coat components as has been shown for coat protein I (COP I), a coat functioning in the early secretory pathway [51]. Alternatively or additionally, they could act indirectly by activating phospholipase D, an enzyme which produces phosphatidic acid from phosphatidylcholine, thereby enhancing the binding of coat components to membranes containing these lipids [52]. *In vitro*, AP-2 binding to membranes is stimulated by the addition of the slowly hydrolysable GTP analogue GTP-γ-S [53,54] or by purified phospholipase D [54], suggesting that its recruitment is controlled by an ARF GTPase that remains to be identified. AP-2 is not recruited to the plasma membrane, however, as would be expected from its role in the plasma membrane derived pathway but, surprisingly, to late endocytic compartments [53,54]. This shows that AP-2 binding sites, probably not functional *in vivo*, can be uncovered on these structures *in vitro*.

The sites to which APs bind with high affinity on membranes probably involve several membrane components. Cargo membrane proteins play some role in AP recruitment. The high level of expression of the transferrin receptor [55,56] or the HIV-1 Nef protein which interacts with selected membrane proteins [46•] increases clathrin coat assembly at the plasma membrane. The expression of the MPRs in cells devoid of these receptors promotes AP-1 recruitment onto perinuclear membranes [39,57]. The over-expression of lysosomal glycoproteins, but not of the MPRs, selectively promotes AP-3 recruitment onto perinuclear membranes [15]. Altogether, these observations support the notion that protein sorting is linked to coat assembly.

In vitro, cytoplasmic domains of membrane proteins usually interact poorly with purified APs, indicating that additional membrane components are required to provide high affinity binding sites for APs. Indeed, phosphoinositides with a phosphate at the D-3 position of the inositol ring enhance the interaction of tyrosine-based sorting signals with the μ2 chain of AP-2 [58] but decrease the interaction of dileucine-based motifs with the β1 subunit of AP-1 [30•]. Thus, the local production of phosphoinositides at distinct membrane sites could regulate the interaction of membrane proteins with APs. Concomitantly, AP subunits could bind directly to membrane lipids. Although this has not yet been demonstrated for APs, support for this notion has recently been provided by studies showing that COP-II-coated vesicles, which mediate endoplasmic reticulum-to-Golgi transport, can bud from chemically defined liposomes incubated with purified coat proteins [59••]. The synthetic budding reaction, however, requires higher levels of COP II proteins than necessary to form vesicles from endoplasmic reticulum membranes [59••], suggesting that these interactions are of a lower affinity. The combination of low affinity interacting components, membrane lipids and proteins coming together in a membrane patch could provide the high affinity AP-binding sites required to produce transport vesicles with a high efficiency.

Conclusions

The past few years have illustrated how APs supporting endocytosis or biosynthetic intracellular transport to endosomes/lysosomes interact with tyrosine- and dileucine-based sorting signals of cargo membrane proteins. Similar types of signals direct membrane proteins through other steps of membrane traffic, such as basolateral sorting in polarized cells or recycling from endosomes back to the plasma membrane [1]. It is likely that other AP-related complexes will be characterized during the next few years.

New types of sorting signals recognized by new types of coats are also emerging. For example, a motif containing two aromatic amino acid residues present in the cytoplasmic tail of one MPR probably controls its endosomal sorting [60].

This motif, also found in other endocytosed proteins, interacts with TIP47, a cytosolic protein [61••]. This potential new coat component is present in a high molecular weight complex, binds to membranes in a GTP γ S-dependent manner and controls the recycling of MPRs from endosomes to the TGN.

Clathrin-coated vesicle biogenesis has been used as a paradigm to illustrate the unidirectional transport of proteins and lipids from one compartment to the next. To determine whether these new coat components follow the same rules for assembly, cargo selection and vesicle formation and how these different types of coats selectively recognize their membranes will be a challenge for the future.

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