

Linking cargo to vesicle formation: receptor tail interactions with coat proteins

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How soluble cargo molecules concentrate into budding vesicles is the subject of intensive current research. Clathrin-based vesiculation from the plasma membrane and the *trans*-Golgi network constitutes the best described system that supports this sorting process. Soluble ligands bind to specific transmembrane receptors which have been shown to interact directly with clathrin adaptor complexes, components of clathrin coats. At the same time, these clathrin adaptors facilitate clathrin coat assembly and probably regulate the recruitment of the rest of the coat components. Recent studies have looked at both the interaction of receptor tails with adaptors and the assembly of the clathrin coat. Progress has also been made in elucidating how soluble cargo molecules may be concentrated for exit from the endoplasmic reticulum.

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Current Opinion in Cell Biology 1997, 9:488–495

<http://biomednet.com/elecref/0955067400900488>

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Abbreviations

AP	adaptor protein
CTLA4	cytotoxic T lymphocyte antigen 4
ER	endoplasmic reticulum
TGN	<i>trans</i> -Golgi network

Introduction

Vesicular traffic between intracellular compartments requires special mechanisms to ensure the selective movement of proteins and lipids from the donor to the acceptor organelle. In general, this problem of selection has been solved by the concentration of specific cargo molecules into vesicles that are formed in a controlled way and which then fuse with the target organelle. The first step in this form of traffic is the binding of cargo molecules to the luminal or extracellular domain of a transmembrane receptor. This is followed by concentration of receptors through interaction with a protein coat that is also required for vesiculation of the membrane. It is likely that cargo concentration and coat formation are linked in order to ensure efficient cargo loading into the assembling vesicles. The vesicles then pinch off from the source membrane in the budding step. Finally, the vesicles are targeted to, and fuse with, the acceptor compartment.

In this review, we focus primarily on the most recent developments in the study of clathrin-coated pits and vesicles, the best understood of the vehicles for moving receptors and ligands from the plasma membrane and the *trans*-Golgi network (TGN) to the endosome. We also suggest some speculative parallels of clathrin-coated vesicles with endoplasmic reticulum (ER)-derived COPII-coated vesicles, major vehicles in ER→Golgi vesicular traffic (see this issue, Kuehn and Schekman, pp 477–483).

Clathrin-coated pits and vesicles

The main structural component on clathrin-coated vesicles is clathrin, a trimeric scaffold protein, which organizes itself into cagelike lattices (reviewed in [1]). Clathrin has the shape of a triskelion, where each one of the three legs is made of a heavy and a light chain. The extended conformation of a clathrin leg allows it to pack along a lattice edge, forming the characteristic open hexagonal and pentagonal facets of the coat. The assembly of a clathrin lattice on the cytosolic side of the plasma membrane or TGN membrane occurs during the formation of a coated pit, and a section of membrane is ultimately captured into a coated vesicle. Clathrin is thus an organizing framework for the proteins that carry out receptor sorting, membrane budding, and other steps in the cycle of vesicle assembly, uncoating and fusion.

The major proteins that drive clathrin coat formation are the 'clathrin AP (adaptor protein) complexes' or 'clathrin adaptors', heterotetramers that couple coated pit assembly to the entrapment of membrane receptors. Endocytic coated pits and coated vesicles contain the AP-2 complex, while coated buds and coated vesicles derived from the TGN contain the related complex AP-1. AP-2 contains two large chains or 'adaptins' (one α chain and one β 1 or β 2 chain), a medium chain (μ 2), and a small chain (σ 2). AP-1 contains the adaptins γ and β 1 together with the medium μ 1 and small σ 1 chains (reviewed in [1–3]). The first hint of the existence of a third AP complex came from the identification in the yeast genome of open reading frames whose sequences were highly related to the known subunit sequences of AP-1 and AP-2 [4,5]. More recently, cDNAs from mammalian sources corresponding to related AP sequences have also been isolated. The newest member of the family is AP-3, a complex found in mammalian cells that contains the δ and β 3 chains together with the smaller μ 3 and σ 3 chains [6*,7*]. This complex, however, is not thought to interact with clathrin.

Clathrin coat assembly

The current view of the recruitment of clathrin coat components to membranes is that APs are first recruited

from the cytosol to the membrane. Little is known about the requirements for recruitment of AP-2 to the plasma membrane. Recruitment of APs to the TGN is influenced by ADP-ribosylation factor (ARF) and GTP γ S [8–10]. The presence of receptor tails, known binding partners for APs (see below), also stimulates AP-1 recruitment to the TGN [10,11]. However, receptor tails are not likely to represent the sole determinant responsible for targeting APs to membranes because the former are known to be present in compartments to which APs are not normally recruited. Therefore, a membrane-bound, high-affinity docking apparatus has been postulated (Figure 1). The identity of the putative AP docking apparatus and how it could work remain unknown. Two studies [12,13] have identified membrane proteins that bind AP-1, but their relevance to AP recruitment has not yet been demonstrated.

The probable next step in clathrin coat assembly is the binding of clathrin to membrane-bound AP complexes. Assembly of the coat is highly coordinated, involving the recruitment of at least 60 clathrin trimers and 20–30 APs. Early evidence based on reconstructed images obtained by electron microscopy of coats suggested that the most distal portion of the clathrin leg, known as the terminal domain, is in contact with APs [14]. The β chains of AP-1 and AP-2 are sufficient to interact with clathrin and drive the formation of coats [15]. These chains of the AP complex contain an amino-terminal core domain and a carboxy-terminal 'ear', which are linked by a hinge. The interaction with clathrin is mediated through the hinge [16]. Phosphorylation of the hinge seems to prevent the association of AP-2 with clathrin and this may be part of the mechanism by which APs initiate and coordinate clathrin coat assembly [17 \bullet]. Evidence for a relatively high-affinity interaction between the α chain of AP-2 and clathrin has also been reported [18] but the biological significance of this association is not clear. Interestingly, AP-3, which is found in clusters associated with endosomal membranes, lacks a recognizable clathrin-binding motif in the β 3 hinge region [19].

A recently discovered component of endocytic clathrin-coated pits and vesicles is Eps15, originally defined as a substrate for phosphorylation by the epidermal growth factor receptor [20]. Eps15 binds to the α chain of AP-2 [21] and colocalizes with clathrin at the plasma membrane [22,23]. The role of Eps15 in coat formation remains to be established. Its carboxy-terminal segment has a binding site for the α ear of AP-2 [21,24]. Its amino-terminal segment contains three Eps homology (EH) domains, modules of 70–90 amino acids that are also present in several yeast proteins including End3p and Pan1p; these proteins are required for endocytosis and organization of the actin cytoskeleton [25–27]. The preferred localization of Eps15 to the rims of coated pits and not in other regions of the coat [22] was unexpected as AP-2, its binding partner, is located throughout the coat. This result suggests

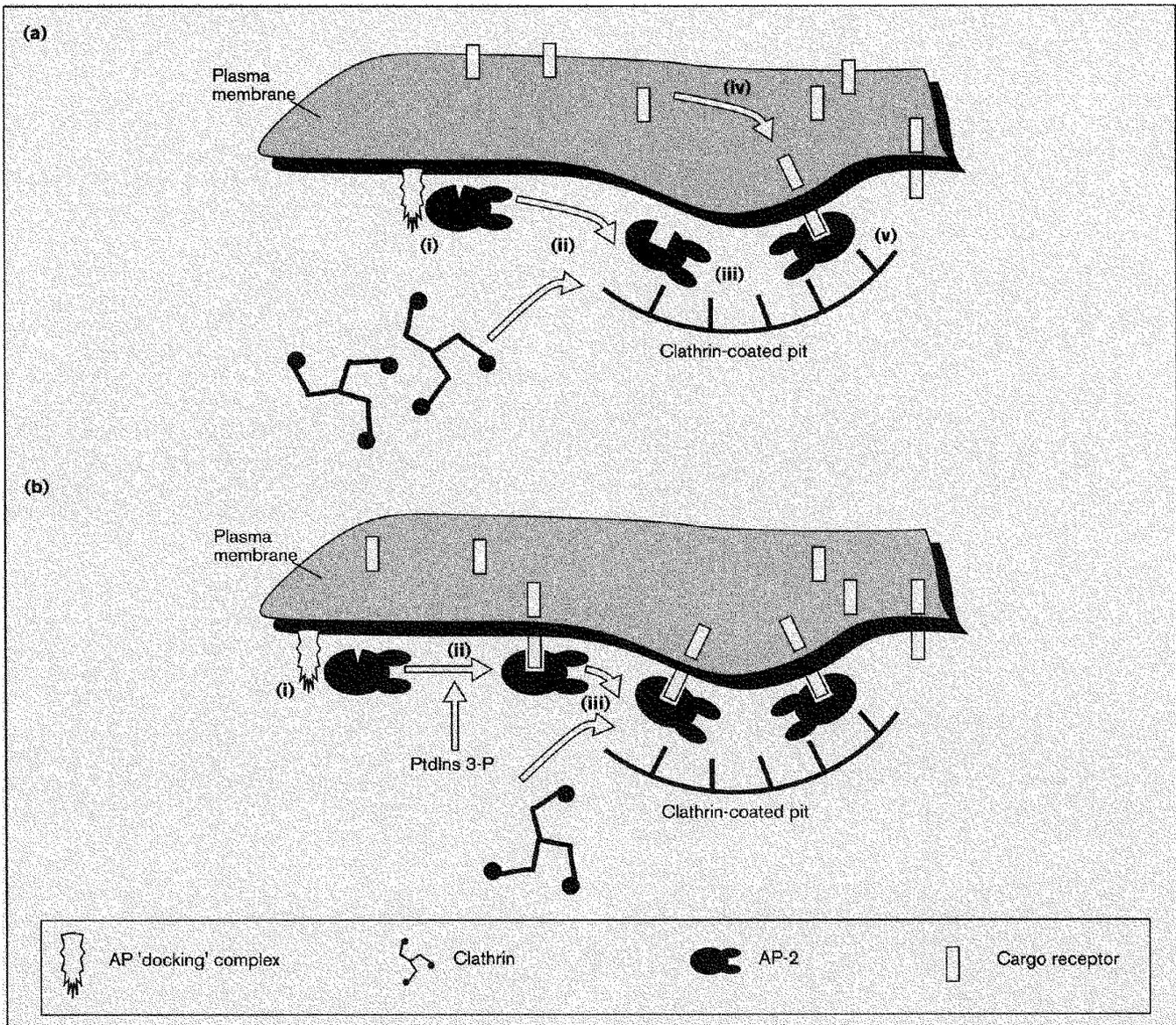
that Eps15 might undergo cycles of binding to and release from APs during coat assembly. Phosphorylation of Eps15 in response to epidermal growth factor stimulation of cells does not affect its intracellular distribution [22]; whether or not the function of Eps15 is linked to signal transduction pathways and receptor downregulation is clearly a question for future studies. Another protein that is highly related to Eps15 has been found [28] and it may be that related proteins are required at different locations of clathrin-coated-vesicle formation.

Internalization signals

The components of clathrin coats are in a position to interact with the cytosolic tails of transmembrane receptors, which have been shown to carry specific signals that direct both their rapid internalization and other intracellular targeting steps. The signals are sequences or structural motifs, many of which have either a critical tyrosine residue or a pair of leucine or bulky hydrophobic residues and are accordingly known as 'tyrosine-based' or 'dileucine-based' signals (reviewed in [29–31]) (see Table 1 [32–38]). There is now extensive evidence indicating that tyrosine-based signals bind directly to the AP-2 complex and that this binding is the event that mediates the concentration of certain plasma membrane proteins within clathrin-coated pits ([39]; reviewed in [31]). Recent experiments suggest that dileucine-based signals also interact with AP-2 [40 \bullet], although they most likely have a binding site different from that of tyrosine-based signals [41 \bullet]. As would be expected for steps dependent on interaction with a limited number of recognition molecules, internalization mediated by both tyrosine-based and dileucine-based signals is a saturable process [41 \bullet]. An important characteristic of both tyrosine-based and dileucine-based signals is that subsets of these signals are involved in additional sorting processes such as targeting to lysosomes, specialized endosomal/lysosomal compartments, the TGN or the basolateral plasma membrane of polarized epithelial cells (reviewed in [30,31]). Thus, some of these signals are likely to be recognized at intracellular sites other than the plasma membrane, probably by adaptor complexes such as AP-1 or AP-3.

One development in the past few years has been the realization that internalization signals are much more diverse than was originally thought. This is true not only of tyrosine-based and dileucine-based signals, which are known to be highly degenerate, but also of an assortment of other cytosolic domain sequences that bear no obvious resemblance to classical internalization signals (Table 1). Signal diversity may be a critical feature of the clathrin coated vesicle sorting machinery. As the ability to concentrate in clathrin-coated pits may not require high affinity, it is possible that even weak interactions of rather nondescript sequences with AP-2 or other components of the clathrin coats may be sufficient to effect internalization. The organization of the coat,

Figure 1



The recognition of tyrosine-based endocytic signals in transmembrane cargo receptors by AP-2 complexes is enhanced by clathrin coats and by 3' phosphoinositides. The figure shows proposed models for the capture of membrane-bound cargo receptors by clathrin-coated pits. **(a)** (i) AP-2 complexes are first targeted from the cytosol to the plasma membrane by interaction with their putative membrane-bound 'docking' complex. (ii) At this point, AP-2 interacts with cytosolic clathrin to form the lattice of coated pits. (iii) AP-2 located in partially formed coated pits displays a clathrin-dependent increase in the affinity of the $\mu 2$ chain of AP-2 for transmembrane receptor tails. (This increase is represented by the change from a triangular to a rectangular 'gap' in AP-2.) (iv) This increase leads to the capture of mobile cargo receptors (v) into the coated pit. It is proposed that the linkage between clathrin binding to APs and the increased affinity of the APs for receptors ensures that coated pit assembly is coupled to receptor sorting. **(b)** (i) AP-2 complexes targeted to the plasma membrane by the putative membrane-bound AP docking complex can (ii) interact with membrane-bound 3' phosphoinositides (PtdIns 3-P), leading to an increase in the affinity of the $\mu 2$ chain of AP-2 for the tyrosine-based endocytic signal located in the cytoplasmic tail of transmembrane cargo receptors. The receptor is shown as bound to AP-2 at this point. (iii) The AP-2-receptor complex recruits cytosolic clathrin to form a coated pit or can be captured by available clathrin already located at the edge of a coated pit. Reproduced with permission from [45*].

with multiple copies of AP-2 being immobilized on a clathrin lattice, and the tendency of some plasma membrane proteins to oligomerize may provide the conditions for the generation of strong avidities from interactions that are weak at a bimolecular level. The combination of diverse internalization signals within the

same cytosolic tail may also allow multivalent attachment to AP-2, thus providing for stronger interactions with the internalization or intracellular sorting machineries. Indeed, tyrosine-based signals, dileucine-based signals and acidic clusters are often found in combinations; the most notable examples of this occurrence are found in the two

Table 1

Internalization signals.			
Signals*	Example of signal sequences*	Proteins containing the signal	References
Tyrosine-based (NPXY-type)	FDNPVY	LDL receptor [†]	[32]
Tyrosine-based (YXXØ-type)	YKYSKV	Cl mannose-6-phosphate receptor [‡]	[33]
Dileucine-based	DKQTL	CD3-γ	[34]
Acidic clusters	WQEECPDSEEDGRGER	Furin	[35]
Dilysine (KKFF-type)	KRFY	VIP36 [§]	[36]
Ubiquitin addition	DAKSS	Yeast α-factor receptor	[37]
Synaptic vesicle targeting	EVVDIMRVNV	VAMP-2/synaptobrevin-2 [#]	[38]

*The single-letter amino acid code is used in these columns. X represents any amino acid and Ø represents a bulky hydrophobic amino acid. Critical residues are shown in bold type. [†]LDL, low-density lipoprotein. [‡]Cl, cation-independent. [§]VIP36, vesicle integral protein 36. [#]VAMP-2, vesicle-associated membrane protein-2.

mannose-6-phosphate receptors which have all three types of signal (reviewed in [42]).

Recognition of tyrosine-based signals by AP-2

Despite the growing diversity of known internalization signals, tyrosine-based signals continue to attract the greatest attention, not only because of historical reasons—they were the first to be discovered—but also because they are the most commonly found among rapidly internalized proteins. Tyrosine-based signals are characterized by the presence of a critical tyrosine residue within an otherwise degenerate sequence context (reviewed in [31]). Extensive analyses of the functional importance of residues neighboring the critical tyrosine residue have established that tyrosine-based signals conform to various sequence motifs, the most common being NPXY and YXXØ (single-letter code for amino acids, where X represents any amino acid and Ø represents a bulky hydrophobic amino acid; see Table 1). Because of their variability, the residues at the Ø and X positions may determine the affinity and fine specificity of the interaction of the signals with the different adaptors and, consequently, may determine the rates of internalization of different proteins as well as the likelihood that the proteins will undergo sorting at some intracellular compartment.

Recent advances in the study of protein–protein interactions have allowed detailed analyses of the specificity of recognition of tyrosine-based signals by clathrin coat components. *In vitro* binding assays have demonstrated a direct interaction of tyrosine-based signals with AP-2 [40*,43,44*]. Screening of cDNA libraries using the yeast two-hybrid system has identified μ2, the medium chain of the AP-2 complex, as a recognition molecule for tyrosine-based signals [43]. This observation was confirmed by various *in vitro* binding assays, including binding of *in vitro* translated, labeled μ2 to glutathione-S-transferase (GST)–tyrosine-based-signal fusion proteins [43] and photoaffinity labeling of the AP-2 complex with tyrosine-based signals [45*]. In addition, μ2 is capable of selecting peptides encoding tyrosine-based signals from combinatorial peptide libraries [46*]. Finally, the fine specificity of interaction of tyrosine-based signals with μ2 correlates with the interaction of tyrosine-based signals with the complete AP-2 complex [46*] as well as with

the known sequence requirements for function of the signals *in vivo* (reviewed in [29,30]). All of these studies have demonstrated that μ2 is capable of binding to many different tyrosine-based signals, although it has a preference for signals that have basic residues at the X positions [46*]. The context in which the signals are found in the cytoplasmic domain also appears to be an important, albeit less predictable, determinant of interactions [47*].

Regulation of the interaction of tyrosine-based signals with AP-2

The entrapment of plasma membrane proteins within clathrin-coated pits can be subject to regulation by modifications of both the tyrosine-based signals and the AP-2 complex. With regard to the tyrosine-based signals, phosphorylation of the critical tyrosine residue has been shown to abrogate interaction with μ2/AP-2 [46*,48*]. This modification is thought to play a role in the regulation of the internalization of the T cell co-receptor cytotoxic T lymphocyte antigen 4 (CTLA4) [48*]. Phosphorylation of other residues outside the signal could modify the local conformational context, making the signals more or less accessible for interaction with AP-2. Another important determinant of interactions could be the oligomeric state of the plasma membrane proteins, as the linking of two or more signals would be expected to increase dramatically the avidity for the immobilized AP-2 complexes. All of these processes could be triggered by binding of ligands to the endocytic receptors, thus providing a means of regulating receptor concentration within clathrin-coated pits on the basis of the occupancy state of the receptors.

Modification of AP-2 might be another way to regulate the recognition of tyrosine-based signals. Binding of AP-2 to clathrin cages, for instance, has been shown to increase the affinity of AP-2 for peptides encoding tyrosine-based signals [45*]. This increase is probably due to a conformational change in the AP-2 complex induced by interaction with clathrin [49]. A consequence of this affinity modulation might be that endocytic receptors are preferentially recruited to AP-2 that is pre-assembled with clathrin (see Figure 1). The AP-2 complex has also been shown to undergo phosphorylation *in vivo* [17*], and it would be interesting to examine whether this phosphorylation affects the recognition of tyrosine-based

signals. The APs are large protein complexes that may respond to additional signals. It has recently been observed that 3'-phosphorylated phosphoinositides enhance the interaction of AP-2 with tyrosine-based endocytic signals [45*]. This finding may help to explain the importance of phosphoinositide 3' kinases in membrane traffic (Figure 1).

β -arrestin as a clathrin adaptor

Until recently, all accumulation of endocytic proteins within clathrin-coated pits was explained on the basis of interactions with AP-2. A study published last year [50*] offers an example of another way of linking endocytic proteins to clathrin lattices. The study shows that binding of agonist to the β_2 -adrenergic receptor causes phosphorylation of the receptor followed by binding of the cytosolic protein β -arrestin to the cytosolic domain of the receptor. β -arrestin then interacts directly with clathrin, leading to concentration of the receptor in clathrin-coated pits and its subsequent internalization. These observations suggest that binding to AP-2 is not an obligatory requirement for concentration within clathrin-coated pits and that other ways of linking receptors to clathrin may be equally capable of mediating internalization. This mechanism may be relevant for other G-protein-coupled receptors and signal transducing receptors that bind cytosolic proteins upon activation. Moreover, it is conceivable that receptors with large cytoplasmic domains may be able to reach into the clathrin lattice without a need for intermediary molecules.

Parallels of clathrin-coated-vesicle transport with transport between the ER and the Golgi

It has been known for several years that different secretory proteins exit the ER at distinct rates. Several attempts to find signals within secretory proteins that could account for their disparate exit rates met with failure. With the discovery of the quality control system in the ER lumen, it was thought that these different transport rates could be explained by retention of proteins due to their differences in processing, folding, or assembly in the ER. However, recently it has become clear that cargo proteins are concentrated in exit sites or vesicles budding from the ER [51,52]. More recently, a new class of small proteins with homology to the yeast protein Emp24p has been found that could play a role in this concentration event.

The primary evidence for such a role came from studies showing that the *emp24* mutation in *Saccharomyces cerevisiae* had a deleterious effect on the ER→Golgi transport of only some secretory proteins. For one of the affected proteins, invertase, it was shown that this defect was not due to a delay in folding or oligomerization. Emp24p was concentrated in ER-derived COPII-coated vesicles; this fact led to the hypothesis that the Emp24p-related proteins act to concentrate cargo molecules into budding vesicles [53]. The *emp24* mutation also caused secretion of Kar2p, a luminal ER chaperone, and could suppress a deletion of the *SEC13* gene which encodes a component of

the COPII vesicle coat [54]. On the basis of indirect data, it was concluded that Emp24p is required for efficient vesicle formation [55], but a subsequent study showed directly that ER membranes without Emp24p formed vesicles efficiently [56*].

Several other members of this family have been found; the first, gp25L, co-purified with calnexin [57]. Other members have been isolated from COPII-coated vesicles [56*] and COPI-coated vesicles [55,58*]. In all cases, the Emp24 family members that have been found are major components of the vesicles, suggesting that they have an important function.

All eight members of the yeast Emp24 family are type I membrane proteins with overall sequence identity of about 20–25%. Two pairs of these sequences (one pair encoded by open reading frames YAR002A and YGL002W, and the other by YAL007C and YOR016C) show much more extensive homology between members of each pair, suggesting overlapping functions. The most variable region is found at the amino-terminal end of the molecule and, as a result of this diversity, could be involved in selective binding of cargo. There are two highly conserved cysteine residues in this domain that are characteristic of protein trafficking receptors, but no direct evidence for cargo binding has yet been obtained. Following this domain is a region of predicted coiled-coil structure which could be involved in a protein assembly reaction. Clear evidence has been presented that at least two members of the yeast family, Emp24p and Erv25p, are functionally and physically associated with each other [56*]. The membrane-proximal portion of the luminal domain is highly conserved, suggesting that it may play a common role among the different members. The transmembrane domains of Emp24 family members are also conserved, and typically contain polar residues. These relatively polar transmembrane domains are likely to be associated with transmembrane domains of other proteins, perhaps other Emp24 family members, to mask these polar residues. Finally, the cytoplasmic tails of these proteins are variations on a common theme. All of them have a conserved glutamine which may be found at the membrane–cytosol interface. The membrane-proximal sequence could form an amphipathic helix, with a hydrophobic residue three residues after the glutamine and one or two conserved phenylalanine residues a turn of the helix later. Three members of the Emp24 family contain a typical dilysine ER-localization sequence of the KXKXX (single-letter code for amino acids) type; four contain basic residues near the carboxyl terminus, but would not be predicted to be efficient dilysine-type signals; and one, Emp24p, contains no basic residues near the carboxyl terminus. The role of the cytoplasmic tail is very likely to be in the binding of cytoplasmic coat proteins (see below), probably both COPI and COPII, as members of the Emp24 family are found in both vesicle populations. If these proteins act as cargo

receptors for traffic between the ER and the Golgi, each one would have to cycle between the two organelles.

Recent experiments have studied the interaction of cytoplasmic tails of different Emp24 family proteins with COPI proteins [59]. This study suggested that the different tail sequences bind to different subcomplexes of COPI. Tails with dilysine motifs bound α , β' , and ϵ COP (B subcomplex), while sequences without the dilysine motif bound β , γ , and ζ COP (F subcomplex). The latter binding was dependent upon the FF (single-letter code for amino acids) sequence in the tails while the former was not. The physiological relevance of these data remains to be shown because the binding efficiency was extremely low (<0.5% of the COP subunits were bound) and because the tails with dilysine signals that bound the B subcomplex also had an FF sequence in a conserved position. Another study [58*] examined the binding of a tail with basic residues that were not in the conserved positions typical of dilysine motifs. Replacement of the basic residues or the FF residues influenced binding to COPI, but the effect was much greater when the FF motif was replaced. No COPI subcomplexes were seen in this study. Comparison of the interactions of the tails with both COP complexes and controlled mutagenesis experiments will be necessary to understand better the role played by these cytoplasmic tails in protein transport. An interesting parallel between the putative coat-binding sequences in these proteins and sorting signals in proteins that bind to adaptins is the possible multiplicity of signals in one tail allowing for several trafficking steps or, possibly, multiple interactions at one step.

Conclusions

In the past few years we have witnessed a significant increase in our knowledge of how cargo molecules, bound to receptors, are concentrated into forming vesicles through interactions of receptor tails with vesicle coat proteins. A diverse set of signals in the receptor tails can mediate this interaction. Even for the best described system, the clathrin-based pathway, the precise sequence of events in vesicle formation still needs to be worked out. During or after receptor-adaptor interaction, the coat is assembled in a coordinated manner. New proteins participating in this process are still being discovered. We are just breaking the surface in our understanding of the regulation of clathrin coat assembly by protein modification and regulatory molecules. A first glimpse suggesting a parallel mechanism for how cargo molecules are transported from the ER comes from the recent discovery of yeast mutations that produce a phenotype expected for a mutation in an ER cargo receptor. However, this work is at a very early stage and more direct experiments will be necessary to examine cargo concentration in this system.

Acknowledgements

The writing of this review was supported by grants to T Kirchhausen (from the National Institutes of Health) and to H Riezman (from the Swiss

National Science Foundation). T Kirchhausen would like to thank the members of his lab for putting up with him while he was having fun writing this review.

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