THREE WAYS TO MAKE A VESICLE

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Cargo molecules have to be included in carrier vesicles of different forms and sizes to be transported between organelles. During this process, a limited set of proteins, including the coat proteins COPI, COPII and clathrin, carries out a programmed set of sequential interactions that lead to the budding of vesicles. A general model to explain the formation of coated vesicles is starting to emerge but the picture is more complex than we had imagined.

ENDOCYTIC PATHWAY
Macromolecules are taken up by invagination of the plasma membrane. They first arrive in early endosomes, then late endosomes, and finally lysosomes, where they are degraded by hydrolases.

SECRETORY PATHWAY
Secretory or membrane proteins are inserted into the endoplasmic reticulum. They are then transported through the Golgi to the *trans*-Golgi network, where they are sorted to their final destination.

FIBROBLAST
Common cell type found in connective tissue in many parts of the body, which secretes an extracellular matrix rich in collagen and other macromolecules and connects cell layers.

MACROPINOCYTOSIS
Actin-dependent process by which cells engulf large volumes of fluids.

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Transport of proteins and lipids along the ENDOCYTIC or SECRETORY PATHWAYS is a hallmark of eukaryotic cells. The membrane fluxes along these pathways are very large and rapid. A FIBROBLAST kept in resting conditions in a tissue culture plate internalizes an amount of membrane equivalent to the whole surface area of the cell in one hour. Inside the cell, it often takes only seconds for a carrier vesicle to move from the donor membrane to the acceptor organelle. But intracellular traffic is not only rapid, it is also very selective. Only a subset of the proteins and lipids in the donor membrane are allowed into the transport vesicle, effectively preventing the homogenization of membrane components and permitting membranous organelles to maintain distinct identities throughout the life of the cell.

Considerable progress has been made towards understanding the molecular basis of membrane traffic[1–8]. A number of traffic pathways have been defined, their major protein components identified and the structures of several of the key components determined at atomic or molecular resolution. The best studied traffic pathways are those that use carrier vesicles that are clearly identifiable by their coats, made of the coatomer COPI, of COPII, or of clathrin and its partners (FIG. 1). During the formation of a vesicle, a limited set of coat proteins (TABLE 1) carries out a programmed set of sequential interactions that lead to budding from the parent membrane, uncoating, fusion with a target membrane and recycling of the coat components. There are clear similarities and differences between the ways that COPI, COPII and clathrin coats handle these steps (TABLE 2).

The first group of reactions, leading to the specific recruitment of coat components to the corresponding donor membrane, form the initiation step. This step is energy dependent and includes sorting of cargo to the forming coat. Coat propagation, the second step in the process, couples further addition of coat components and additional recruitment of cargo with invagination of the underlying membrane. When formation of the coat ends, the vesicle buds by scission of the neck connecting the deeply invaginated membrane to the donor surface. This is a relatively simple step for COPI- and COPII-coated vesicles but involves a significant amount of regulation for clathrin-coated vesicles. Finally, during uncoating, the coat components are released so that membrane fusion can occur between the naked vesicle and the target organelle. This article discusses the emerging molecular rationale for coated-vesicle assembly for, in order of increasing complexity, COPII, COPI and clathrin.

Pathways of membrane traffic

COPI and COPII vesicles traffic between the endoplasmic reticulum (ER) and the Golgi complex — COPI primarily from the Golgi to the ER and between Golgi cisternae, and COPII from the ER to the Golgi (FIG. 1). The clathrin pathway has two major routes, from the plasma membrane to the early endosome and from the Golgi to the endosome. Other structures have been observed in the cell that do not have COP or clathrin coats. Internalization from the plasma membrane can also occur via MACROPINOCYTOSIS, PHAGOCYTOSIS and probably through CAVEOLAE[9]. In the secretory pathway, poorly understood tube-like structures connect the Golgi with the plasma membrane and the ER[10–13]. In the endocytic pathway, tubules emerge from early endosomes and participate in recycling to the plasma membrane[14]. These carrier structures have been hard to study, partly because they tend to be heterogeneous. In some cases,

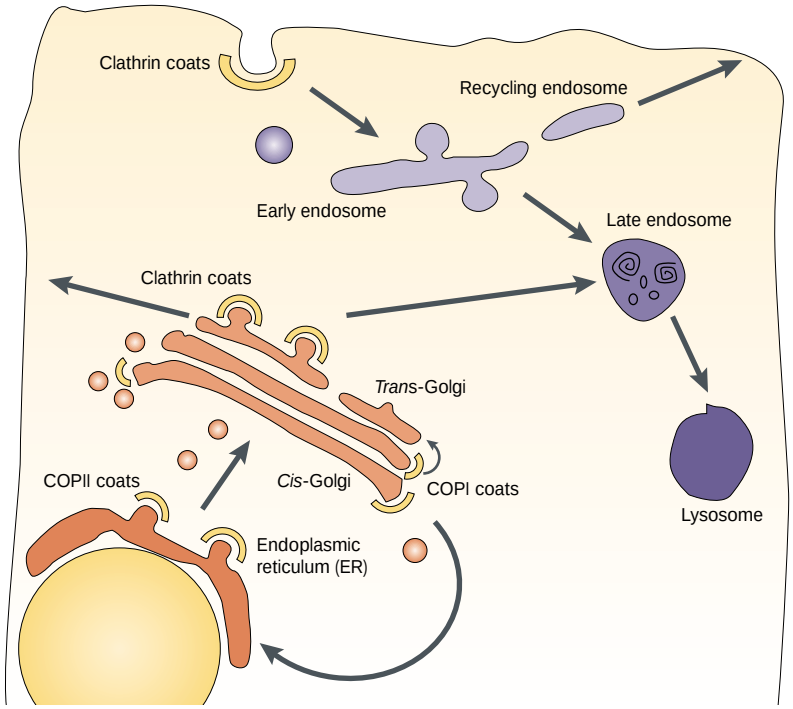

Figure 1 | **The major membrane traffic pathways that use carrier vesicles coated with COPI, COPII and clathrin in eukaryotic cells**. In the biosynthetic pathway, newly synthesized molecules are transported from the endoplasmic reticulum to the Golgi and from one cisterna of the Golgi to the next until they reach the *trans*-Golgi network. There, sorting occurs, directing traffic to the plasma membrane or to endosomes. In the endocytic pathway, macromolecules are internalized at the plasma membrane and forwarded to early endosomes, from where they are either recycled to the plasma membrane through recycling endosomes or forwarded towards degradation in late endosomes and lysosomes.

they might be used for large-scale movement of selectively captured membrane components[12,15]. The most important distinction between coated vesicles and other forms of membrane carriers is the presence of an identifiable protein coat, which assembles at a particular region of the membrane, locally deforming it. Although the events required are similar — tubes, macropinosomes and phagosomes also need to form, pinch off, reach their target and fuse with it just like coated vesicles — it is impossible to transfer our understanding of vesicle traffic to these other mechanisms.

COPII

COPII components, and COPII-coated vesicles, were originally discovered in the yeast *Saccharomyces cerevisiae* using genetic approaches coupled with a cell-free assay that measured the transfer of a marker protein from the ER to the Golgi[3,8]. This pathway has a mammalian counterpart and most of the COPII components have been identified (TABLE 1). Newly synthesized proteins destined for secretion are sorted into COPII-coated vesicles at specialized regions of the ER, which in mammalian cells do not contain membrane-bound ribosomes.

Biochemical analysis has led to a relatively detailed model for the mechanism of COPII vesicle formation. But as there is no structural information on any of the coat components, it is hard to claim that we understand the mechanism of even this relatively simple pathway in molecular detail. Nevertheless, there is good agreement in the field that the major components have been identified and there is a good working model for the sequence in which they act (FIG. 2).

Biochemical and cell biological observations. Although several as-yet-undefined membrane components are presumably necessary for efficient operation of the COPII pathway, vesicular transport can be minimally reconstituted using three cytosolic components containing a total of five proteins: the Sec23p–Sec24p complex, the Sec13p–Sec31p complex and the small GTPase Sar1p (REF. 16). These proteins support a cargo-carrying budding reaction from isolated ER membranes. Deletion of the genes for most of these components is lethal for yeast, highlighting the strict dependence on this pathway for ER-to-Golgi traffic.

The events driven by these five cytosolic proteins, together with the membrane components required for targeting and fusion, are varied and complex (TABLE 2). Cargo capture, deformation of the budding membrane, scission to detach the forming vesicle from the donor membrane and coat release before fusion of the vesicle with the Golgi network all occur in this minimal system. The GTP-binding protein Sar1p is particularly important for the budding reaction because its activation initiates coat formation; it also recruits part of the 'label' needed for correct vesicle targeting and fusion. The GDP-bound form of Sar1p is normally cytosolic and is recruited to the ER membrane upon interaction with Sec12p, an ER-bound membrane protein that serves as the guanine exchange factor (GEF) for Sar1p (REF. 17). Sar1p–GTP then facilitates the association of the Sec23p–Sec24p complex with cargo proteins.

The Sec23p–Sec24p complex is probably the component responsible for cargo recognition[18,19] but the sorting signals recognized by the complex remain to be identified. Members of the p24 family of transmembrane proteins bind to Sec23p through a cytosolic diphenylalanine motif. As these proteins are required for efficient ER-to-Golgi traffic of some cargo proteins[20], it is thought that they might serve as cargo adaptors[21]. In addition to recruiting the Sec23p–Sec24p complex, the GTP-bound form of Sar1p activates Sec23p to bind SNARE proteins involved in the specificity of targeting and in the fusion reaction of vesicles with acceptor membranes[18]. ER membranes with Sec23p–Sec24p and Sar1p can then recruit Sec13p–Sec31p (REF. 16). The complex is likely to act as a scaffold, very much like clathrin, to drive membrane deformation and to complete vesicle budding. Completing the cycle, Sec23p acts as a GTPase-activating protein (GAP) for Sar1p. It is thought that, after GTP hydrolysis, Sar1p–GDP is released, leading to uncoating before fusion of the vesicle to the target membrane and formation of a new coated vesicle. GTP hydrolysis by Sar1p is thus a timer, triggering uncoating at a suitable interval after coat formation.

Studies on live cells. The model describing the formation of COPII vesicles brings together a number of bio-

PHAGOCYTOSIS
Actin-dependent process, by which cells engulf external particulate material by extension and fusion of pseudopods around each particle.

CAVEOLA
Flask-shaped invagination at the plasma membrane, possibly involved in the uptake of extracellular materials.

Table I | Coat proteins and their main binding partners

Protein	Mammalian	Yeast	Salient features
COPII			
Sar1p	hSar1p	Sar1p	Small GTPase; Ras family
Sec13p–Sec31p	hSec13p hSec31p	Sec13p Sec31p	WD 40 repeats (β-propeller) WD 40 repeats (β-propeller)
Sec23p–Sec24p	hSec23p hSec24p ? ?	Sec23p Sec24p/Iss1p/Lst1p Sec12p Sec16p	Sequence homology with Sec24p; GAP for Sar1p Sequence homology with Sec23p GEF for Sar1p Membrane protein; forms a ternary complex with Sec23p–Sec24p
COPI			
ARF1	ARF1	yARF1/2/3	Small GTPase; Ras family
Coatomer	α-COP β-COP β′-COP γ-COP δ-COP ε-COP ζ-COP	Ret1p Sec26p Sec27p Sec21p Ret2p Sec28p Ret3p	WD 40 repeats (β-propeller) Binds ARF1 weak sequence identity to β-AP WD 40 repeats (β-propeller) Binds members of p24 family Weak sequence identity to μ-AP Weak sequence identity to σ-AP
ARFGAP	ARFGAPs	Glo3p	GAP for ARF
ARFGEF	ARFGEFs	Gea1p/Gea2p	GEF for ARF
Clathrin			
Clathrin	HC LCa/b	Chc1p Clc1p	Subunits polymerize into a triskelion; Atomic structures for several fragments are known: α-zigzags and β-propeller Unknown function
Adaptors			
AP-1	γ β1 μ1 σ1	Apl4p Apl2p Apm1p Aps1p	γ-AP, β-AP and α-AP sequences are related σ1 sequence weakly related to N-terminal portion of μ's
AP 2	αa/c β2 μ2 σ2	Apl3p Apl1p Apm4p Aps2p	Atomic structure of C-terminal α-ear is known Atomic structure of C-terminal β-ear is known Atomic structure of part of μ2 interacting with YppØ sorting motifs is known σ2 sequence weakly related to N-terminal portion of μ's
β-arrestin	β-arrestin1/2	None	Atomic structure not solved but probably similar to known α-arrestin structure
Partners			
Amphiphysin	Amphiphysin	Rvs161p/167p	Binds clathrin, AP-2, dynamin
AP180	AP180	yAP180	Binds clathrin; regulates size of neuronal vesicles
ARF1	ARF1	Arf1p/2p	Helps recruit AP-1
Auxilin	Auxilin1/2	Aux1p	Contains J domain and is cofactor for Hsc70 uncoating ATPase; binds clathrin
Dynamin	Dynamin		Large GTPase; fission of necks in deeply invaginated clathrin pits
Endophilin	Endophilin		Fission; membrane deformation by changes in lipid composition at neck of deeply invaginated clathrin pit
Epsin	Epsin	Ent1p/2p	
Eps15	Eps15/15R	Pan1p	Binds AP-2; located at rim of clathrin coated pits; mainly excluded from clathrin coated vesicles
GGA	GGA	Gga1p/Gga2p	Binds γ-synergin; traffic regulation from Golgi to lysosome/vacuole
Intersectin	Intersectin1/2		
Synaptojanin	Synaptojanin	Sjl1p/3p	Phosphoinositide 5′-phosphatase; role in coat release
Synaptotagmin	Synaptotagmin	None	Ca^{2+} sensor; binds AP-2
Syndapin I	Syndapin I	None	Binds N-WASP; link to actin network/signalling cascade ?
Uncoating ATPase	Hsc70	Ssa1p/2p	Dissociation of clathrin from coats
γ-synergin	γ-synergin		Binds AP-1; unknown function

(AP, adaptor protein; ARF, ADP-ribosylation factor; ARFGAP, ADP-ribosylation factor GTPase activating protein; ARFGEF, ADP-ribosylation factor guanine exchange factor; COP, coatomer protein; Eps15, EGF receptor pathway substrate clone 15; GAP, GTPase activating protein; GEF, guanine exchange factor; N-WASP, neuronal Wiscott–Aldrich syndrome protein.)

GREEN-FLUORESCENT PROTEIN
Autofluorescent protein originally identified in the jellyfish *Aequorea victoria*.

chemical and genetic observations. The first efforts to visualize the budding of COPII vesicles in live cells yielded surprising results. The stably expressed human homologue of Sec13p and the transiently expressed human homologue of Sec24p, both tagged with the GREEN-FLUORESCENT PROTEIN (GFP), were detected by time-lapse fluorescence microscopy[22,23]. They were found in stable and nearly immobile bright spots associated with ER sites devoid of ribosomes, presumably where COPII vesicles form and bud.

It is difficult to reconcile the stability of these spots with a model in which the recruitment of cytosolic Sec13p to the ER membrane is concurrent with formation and budding of COPII-coated vesicles. It is possible

Table 2 | Steps in coat formation

	COPII	*COPI*	*Clathrin*
I. Initiation			
1. GTPase activation and membrane binding	Sar1p–GDP is recruited to ER by transmembrane GEF (Sec12p) and converted to Sar1p–GTP	ARF1– GDP is recruited to Golgi by soluble GEFs (Gea1p and Gea2p), probably bound to Golgi membranes, and is converted to ARF1–GTP	*TGN* ARF1–GTP recruited to *trans*-Golgi membrane; mechanism is unknown *Plasma membrane* ?
2. Cargo and v-SNARE recruitment	a. Sar1p–GTP recruits Sec23p–Sec24p complex b. Sec23p–Sec24p binds to members of the p24 protein family of possible cargo receptors, and together with Sar1p binds v-SNAREs (Bet1p, Bos1p)	a. ARFGAP binds to ARF1–GTP and to the transmembrane KDEL-receptor b. ARF–GTP together with cargo proteins containing C-terminal KKXX (or KXKXX) motifs recruit cytosolic COPI coatomer	*TGN* a. ARF1–GTP recruits AP-1 adaptor to *trans*-Golgi membrane b. AP-1 binds to membrane receptors containing YppØ and LL motifs *Plasma membrane* a. ATP, GTP and phosphoinositides required to recruit AP-2 to plasma and/ or endosomal membranes; mechanism is unknown b. AP-2 binds to membrane protein synaptotagmin c. AP-2 binds to membrane receptors containing YppØ and LL motifs
3. Start of coat assembly	Cytosolic Sec13p–Sec31p complex binds to pre-bound Sec23p–Sec24p		Cytosolic clathrin recruited to pre-bound AP-1 or AP-2 adaptors
II. Propagation			
1. Loss of GTPase	Sar1p–GTP hydrolysis increased15–30-fold by Sec23p; Sar1p–GDP released and used in further cycles	ARF–GTP hydrolysis increased 1,000-fold by ARFGAP and COPI; ARF–GTP released and used in further cycles Hydrolysis rate depends on sequence of C-terminal motif in cargo	Not known
2. Further cargo recruitment and coat assembly	Growth of coats by sequential incorporation of other coat elements Membrane-bound cargo proteins diffuse laterally and are captured by the forming coats		*Plasma membrane* a. Association of AP-2 with clathrin or with phosphoinositides increases the affinity of AP-2 for YppØ motifs b. β-arrestin recruits seven-transmembrane G-coupled receptors to clathrin coats c. Growing edge of clathrin lattice contains AP-2 bound to Eps15
3. Membrane deformation	Continuous process that is coupled to the growth of the coat		
III. Vesicle budding			
	No other proteins are required *in vitro*; energy for membrane scission provided by coat polymerization	No other proteins are required *in vitro*; energy for membrane scission provided by coat polymerization	a. Amphiphysin binds to clathrin and AP-2 and acts as a dynamin receptor b. Dynamin–GDP is recruited to the neck of the budding vesicle and polymerizes into a dynamin ring; dynamin–GEF is unknown c. Endophilin is recruited to the ring. Membrane deformation and fission is facilitated by the coupling of the acyl transferase activity of endophilin and the neck constriction imparted by the dynamin ring d. Dynamin (in the rings) acts as its own dynamin–GAP; GTP hydrolysis releases dynamin for another cycle
IV. Uncoating			
	Spontaneous ? (following GTP hydrolysis of Sar1p)	Spontaneous ? (following GTP hydrolysis of ARF1 activated by ARFGAP at time of cargo recognition or by second ARFGAP located close to the target membrane)	*Plasma membrane* a. Hsc70–ATP and auxilin bind to clathrin coats and drive disassembly, presumably by a clockwise twist imparted on clathrin triskelions (regulation exists but the mechanism is unknown) b. A fraction of AP-2 can be dissociated from membranes by Hsc70–ATP and an unknown cytosolic factor c. Phosphorylation of AP-2 (β-subunit) prevents its association with clathrin d. Synaptojanin required for efficient release of coats

(AP, adaptor protein; ARF1, ADP-ribosylation factor 1; ARFGAP, ADP-ribosylation factor GTPase activating protein; Eps, EGF receptor pathway substrate; GAP, GTPase activating protein; GEF, guanine exchange factor.)

that Sec13p and other COPII components accumulate in specific regions of the ER membrane, which can then act as reservoirs for the subsequent assembly of a COPII coat with the curvature needed for membrane fission and vesicle budding. The relatively low expression level of the described hSec13p–GFP would have made the detection of single COPII vesicles (as opposed to extended reservoirs) difficult. The development of algorithms and detectors that can distinguish weak signals from background noise and the generation of stronger chromophores would much improve our ability to analyse the dynamics of vesicular traffic.

COPI

COPI-coated vesicles occur in various intracellular contexts[1] and this versatility of function seems to correlate with a greater biochemical complexity compared with COPII vesicles (TABLE 1). Whereas COPII-dependent traffic is unidirectional (from ER membranes to the Golgi), the direction of COPI traffic is still a matter of controversy. COPI-coated vesicles seem to function primarily in retrograde transport from the ER–Golgi intermediate compartment to the ER but they are also important in forward transport within the cisternae of the Golgi.

The COPI coatomer is a complex of seven proteins (α, β, β′, γ, δ, ε and ζ). COPI-coated vesicles efficiently capture proteins carrying in their cytoplasmic carboxy-terminal domain sorting signals of the form KKXX (the dilysine motif) or KXKXX (X is any amino acid). The KDEL receptor, a multiple-spanning membrane protein that binds and retrieves lumenal proteins containing the KDEL carboxy-terminal sequence, is also transported along this pathway. The γ subunit seems to be the component responsible for cargo recognition because it recognizes the KKXX and KXKXX motifs, but it is not known whether it also recognizes the KDEL receptor[24]. Members of the p24 protein family also interact with COPI coatomers in addition to COPII and might facilitate the recruitment of COPI coatomers to Golgi membranes[25].

The initial event in the COPI pathway that leads to recruitment of the coat requires the association of the

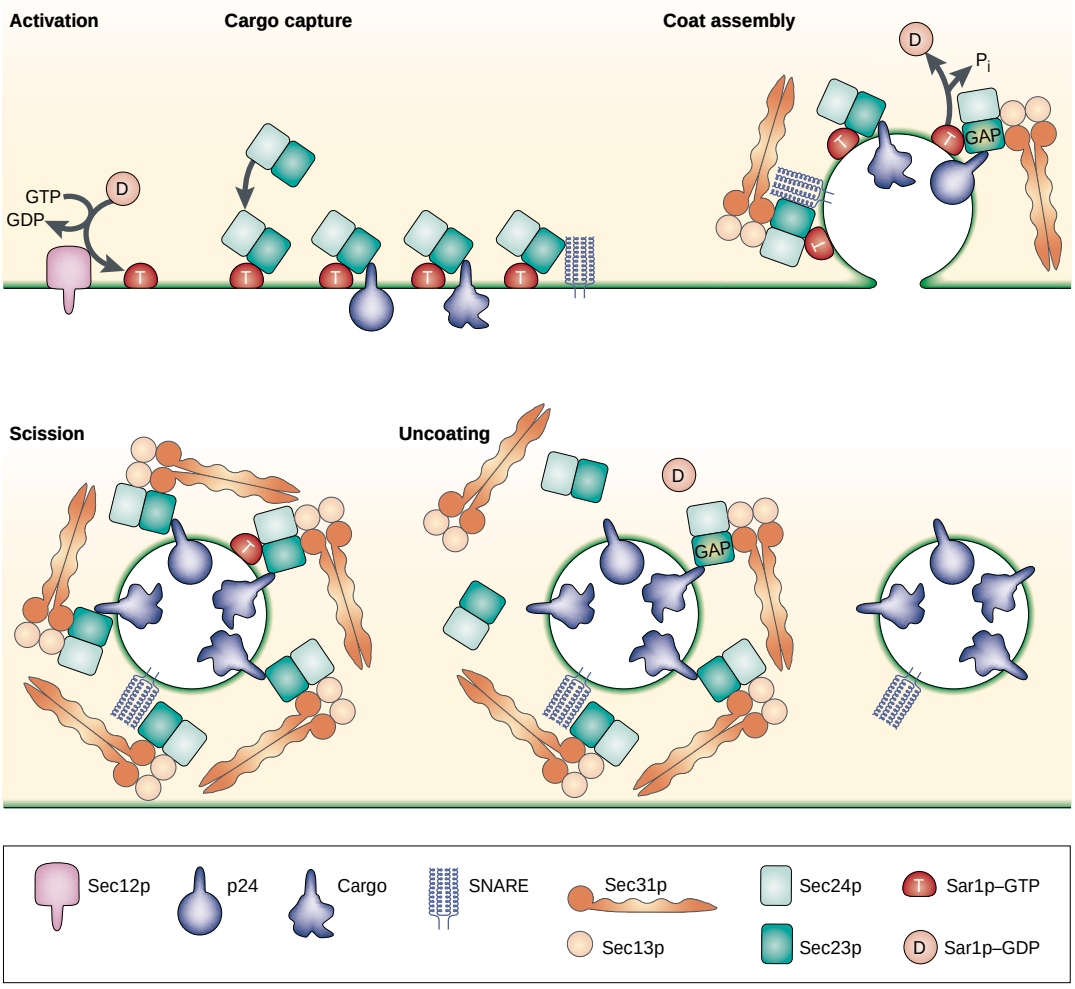

Figure 2 | **The key steps in the formation of COPII-coated vesicles.** Coat assembly is activated by the recruitment of Sar1p–GTP to the membrane. This allows the binding of the Sec23p–Sec24p complex and the recruitment of cargo. The Sec13p–Sec31p complex binds next, leading to membrane deformation. When the coat is complete, the vesicle buds. The GTPase activity of Sar1p is enhanced by Sec23p, which acts as a timer, leading to inactivation of Sar1p and uncoating. (GAP, GTPase activating protein.)

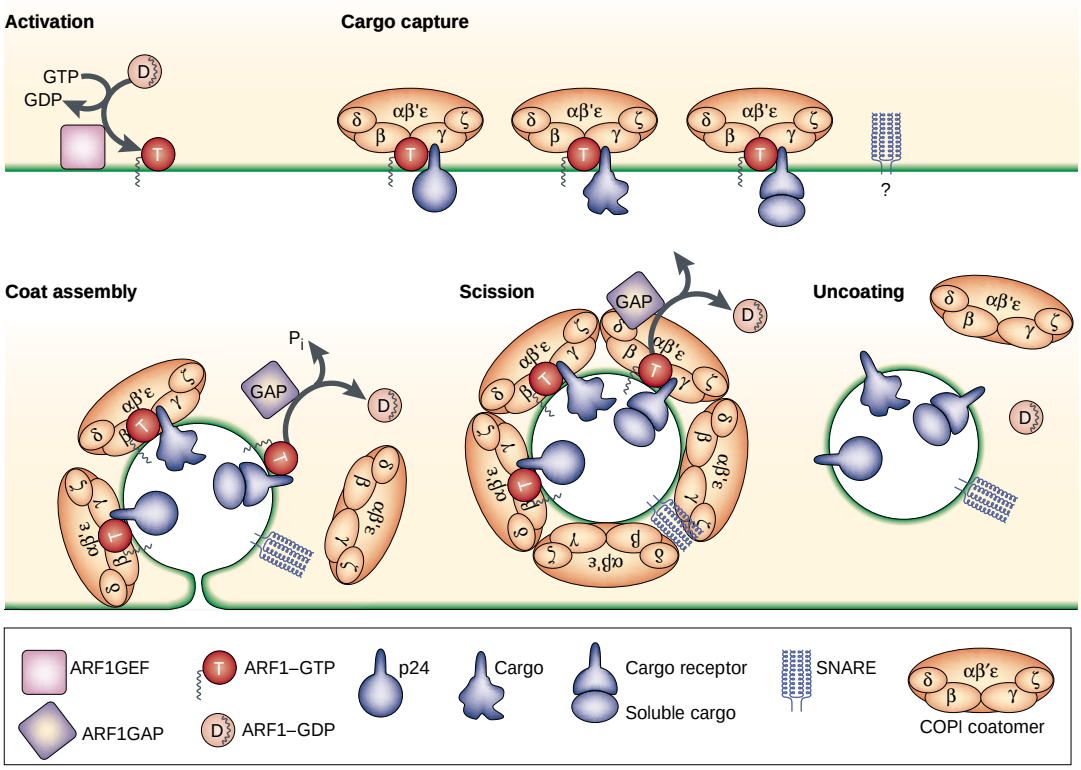

Figure 3 | **The key steps in the formation of COPI-coated vesicles.** Coat assembly is activated by the recruitment of ARF1–GTP to the membrane. This allows the binding of the COPI coatomer and the recruitment of cargo. GTP hydrolysis is slow when ARF1 is bound to its preferred cargo, allowing kinetic regulation of coat recruitment. Membrane deformation occurs at the same time as coat recruitment. When the coat is complete, the vesicle buds. The GTPase activity of ARF1 is enhanced by ARF1GAP, which acts as a timer, leading to inactivation of ARF1 and uncoating. (ARF1, ADP-ribosylation factor 1; ARF1GAP, ADP-ribosylation factor 1 GTPase activating protein; ARF1GEF, ADP-ribosylation factor 1 guanine exchange factor.)

GTPase ARF1 (ADP-ribosylation factor 1) in its active form to the membrane (FIG. 3, TABLE 2). The ARF protein family has many members and targeting of ARF1 to the correct membrane involves specific association with its appropriate GEF. Several GEFs for ARF1 have been identified, one of which (known as ARF1GEF, ARNO3 or GRP1) seems to be specifically associated with the COPI pathway[26]. In contrast to the COPII-associated Sar1p, ARF1 is MYRISTOYLATED to allow its membrane association. In the GTP-bound state, the myristoyl group is exposed and ARF1 becomes membrane bound. When the GTP is hydrolysed, the protein undergoes a conformational change, developing a myristoyl-binding pocket that covers the tail, solubilizing the protein. It has been suggested that the hydrolysis of GTP and release of ARF1 from the membrane act as a timer, triggering (as with Sar1p and COPII vesicles) the release of the other coat components and preparing the vesicle for fusion with its target membrane.

The rate at which ARF1 hydrolyses GTP depends on its association with ARFGAP and the COPI complex[27], both of which are required for full GTPase activation. An element of further regulation has recently been uncovered in this step. A synthetic peptide containing the carboxy-terminal FFXXRRXX sorting signal of the p24 protein hp24a, which binds to COPI, markedly reduces the ability of COPI to stimulate ARFGAP[28]. However, other peptides that contain the FFXXKKXX sequence and bind to the same site in COPI do not affect the stimulation of GTP hydrolysis of ARF by COPI (FIG. 3). These data indicate that the inhibitory peptides may represent preferred cargo. When these peptides are present on a protein tail, the rate of GTP hydrolysis will be slow even when some COPI has been recruited to the membrane. Vesicles that capture preferred cargo will retain their ARF1 protein long enough to complete coat assembly, whereas vesicles that capture other proteins will not. This proposal suggests that cargo selection is kinetically regulated: it depends not on different affinities of cargo for the coat but instead on a dynamic regulation of the rate of coat release.

Although there is substantial confidence that most of the major components of this pathway have been identified, there is still some way to go before we understand the functions of each component. The structural characterization of the components of the pathway is just beginning — the crystal structures of ARF1 and ARF1 complexed to a domain of GAP have been determined but no part of the COPI complex has yet been visualized. Structures for these components are likely to emerge before long.

MYRISTOYLATION
Covalent attachment of a hydrophobic myristoyl group to the amino-terminal glycine residue of a nascent polypeptide.

Clathrin

Clathrin-coated vesicles are the most prominent of the carrier vesicles and were the first to be discovered and

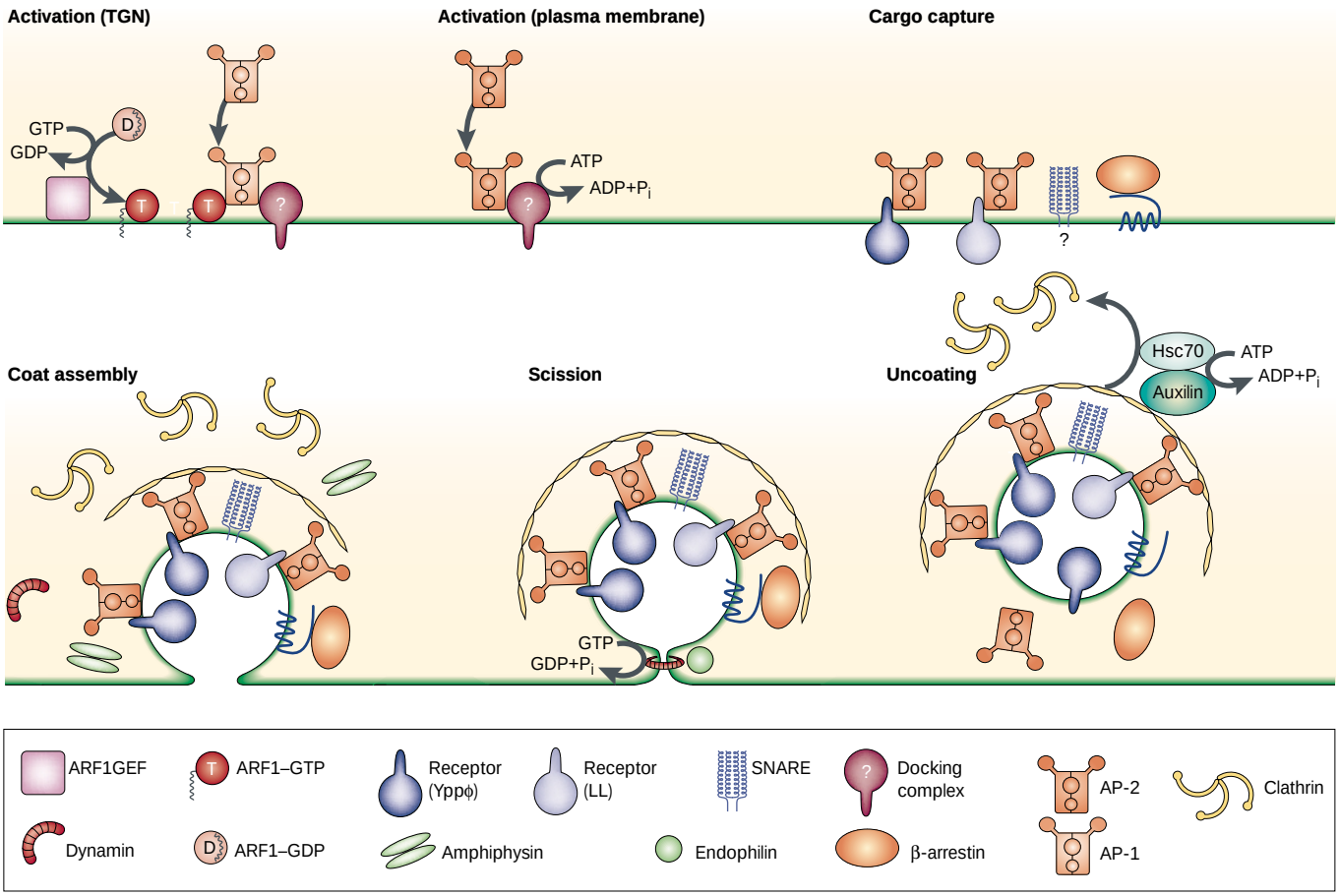

Figure 4 | **The key steps in the formation of clathrin-coated vesicles.** At the *trans*-Golgi network, coat assembly is activated by the recruitment of ARF1 to the membrane. It is not clear how coat assembly is activated at the plasma membrane. One end of adaptor proteins bind to cargo molecules and the other end to other coat components, including clathrin. Clathrin triskelions polymerize into hexagons and pentagons, forming a cage, which leads to membrane deformation. When the coat is almost complete, dynamin (together with accessory proteins) pinches off the vesicle. Uncoating requires ATP hydrolysis by Hsc70 and auxilin. (AP, adaptor protein; ARF1, ADP-ribosylation factor 1; ARF1GEF, ADP-ribosylation factor 1 guanine exchange factor.)

studied[5,29–31]. The low-density lipoprotein (LDL) receptor and many other plasma membrane proteins and their ligands are internalized by the clathrin pathway for traffic to endosomes, and clathrin-coated vesicles bud from the *trans*-Golgi network (TGN) to fuse with endosomes. Yeast cells also use a clathrin-based pathway for membrane traffic, although its use in the secretory pathway seems to be more prominent than its role in endocytosis[32–35].

Recruitment of the clathrin coat. Clathrin is the most abundant protein in the coat of these vesicles and it provides the scaffold that orchestrates protein sorting, membrane deformation and budding[5,36,37]. By contrast to the COPI and COPII vesicles, clathrin-coated vesicles have a large variety of associated proteins (TABLE 1). So far, more than 25 proteins have been identified as partners in the endocytic pathway alone[5,38]. The functions of most of these proteins still have to be elucidated (TABLE 2). Vesicles that form at the TGN carry heterotetrameric AP-1 adaptor protein complexes, whereas vesicles that form at the plasma membrane carry related AP-2 complexes. AP-1 and AP-2 adaptors bind to certain sorting signals (FDNPVY, tyrosine-based YppØ motifs and dileucine motifs) found in the cytosolic tails of a large number of membrane proteins. The non-visual arrestins are a second type of adaptor, which recruits seven-transmembrane-helix G-protein-coupled receptors at the plasma membrane to clathrin-coated vesicles. This occurs by direct contacts with the cytosolic side of the G-protein-coupled receptors, with clathrin and with AP-2 (REFS 39–41). Adaptors also interact with a number of other proteins involved in clathrin-coated-vesicle function[4], and they recruit clathrin to the membrane, initiating coat formation.

As with the COPI and COPII systems, the recruitment of clathrin coat components to the appropriate target membranes is nucleotide dependent (FIG. 4). The role of ARF1 in the clathrin pathway is similar to, but not the same as, its role in the COPI pathway. In both cases, ARF1 helps to recruit key coat components but, in the clathrin pathway, it is involved only in the recruitment of AP-1 to Golgi membranes[42–45]; ARF1 does not seem to act as a timer for uncoating as it does for COPI. *In vitro* recruitment of AP-2 to the plasma membrane, endosomes and lysosomes also requires the activation of cytosolic components by ATP and GTP, but the identity of the

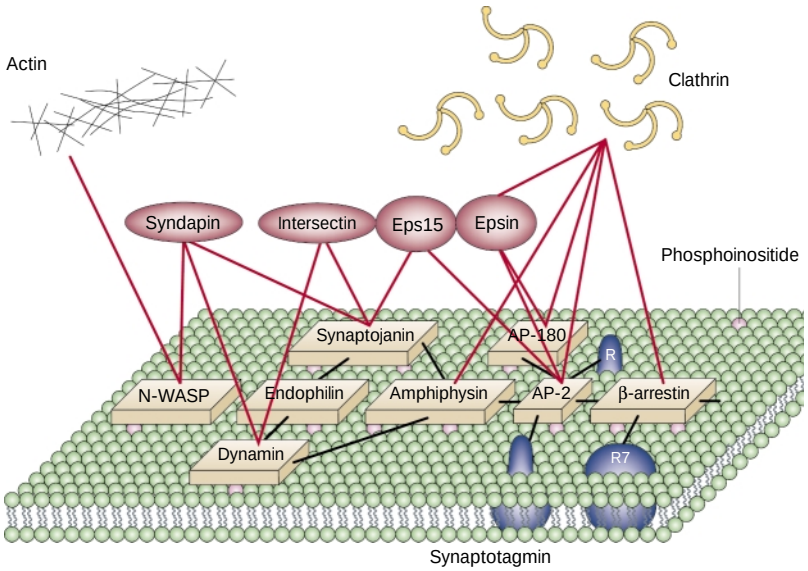

Figure 5 | **Network of protein–protein and protein–lipid interactions involved in clathrin coat formation at the plasma membrane.** Proteins that bind to phosphoinositides are represented here in direct contact with the plasma membrane, although this interaction might be transient. Often, two or more interactions have been detected, leading to the idea that these proteins might operate as coincidence detectors. (AP, adaptor protein; Eps15, EGF receptor pathway substrate clone 15; N-WASP, neuronal Wiskott–Aldrich syndrome protein; R, transmembrane receptor; R7, seven-transmembrane-helix G-protein-coupled receptor.)

nucleotide-binding or -hydrolysing protein(s) is still obscure[46,47]. It has been proposed that the membrane protein synaptotagmin, a Ca^{2+} sensor, is required for AP-2 recruitment, perhaps as part of a set of postulated docking proteins required for efficient recruitment[48]. The interaction between AP-2 and synaptotagmin is nucleotide independent but it is enhanced by peptides bearing the YQRL tyrosine-based endocytic signal[48]. As synaptotagmin is primarily found in brain tissues, its role in clathrin-coat formation might be more as a coincidence detector than as a general docking protein.

A simple model for clathrin-coat formation, which covered most of the evidence until very recently, was that the AP complexes have two essential functions: they bind to certain sorting motifs in receptor tails and they recruit clathrin to the membrane to initiate coat formation. Although this model is still logical and plausible, two new reports that genetic knockouts of all of the AP proteins in yeast do not show the expected profound defects in vesicle formation or membrane traffic force a re-examination of its details[49,50]. If the AP proteins are performing the functions of cargo selection and coat initiation at all, there must be a second protein or set of proteins available to take over these functions when required. Genetic knockouts of μ- or α- subunits of the AP proteins in multicellular organisms such as *Drosophila melanogaster*[51] and *Caenorhabditis elegans*[52,53] are not lethal, whereas, in mice, knockouts of the μ1 or γ- subunits of AP-1 are embryonically lethal[54,55]. These data indicate that the severity of the deletion is compounded by the cellular context in which AP function is hindered. The unexpected outcome of some of these genetic experiments should be taken as a cautionary note that the biochemical studies used to propose models are still not sufficient to take into account all of the functions exerted by these coat proteins.

Regulation of vesicle formation. Amphyphysin[56], epsin[57,58], synaptojanin[59] and Eps15 (EGF receptor pathway substrate clone 15)[60,61] are some of the many accessory proteins found in association with clathrin coats at the plasma membrane. They not only interact with clathrin but also have binding sites for AP adaptors, for proteins such as the large GTPase dynamin (itself involved in the budding step) and even for specialized lipids such as phosphoinositides. Although the functions of many of these proteins are still not known, it is becoming increasingly clear that they are part of a network of complex molecular switches and contacts that regulate various aspects of clathrin-mediated traffic (FIG. 5). On the basis of biochemical characteristics, many of these proteins seem to function as coincidence detectors, needing two or more simultaneous relatively weak interactions to exert their function. For example, AP-2 and β-arrestin have binding sites for clathrin, for cargo recruitment and for each other[41,62–66]. Simultaneous interactions probably occur between these molecules when they are recruited to the clathrin coat, providing a control point for synchronizing the concentration of cargo into endocytic vesicles.

Uncoating. The heat shock protein Hsc70 and auxilin, a J-DOMAIN-containing protein, are responsible for clathrin disassembly[67–70] through an ATP-dependent reaction, which is presumably subject to regulation. The uncoating mechanism is therefore fundamentally different in the COPI or COPII and clathrin pathways. In both COP pathways, uncoating results from a change in the properties of coat components in response to GTP hydrolysis in Sar1p or ARF1. In the clathrin pathway, Hsc70 and auxilin, proteins that do not participate in the process of coat assembly, are required to achieve the same effect. The most plausible reason for this difference is a requirement for independent control over various steps in the clathrin pathway and over various distinct uses of clathrin vesicles.

Vesicle fission. The mechanism of vesicle fission also seems to be more complex in the clathrin pathway. In the COPI and COPII pathways, vesicle fission is intrinsic to the completion of coat assembly. Deformation and fission of the membrane requires energy and, for the COPs, this energy comes primarily from the energy of association of the coat proteins as the coat forms. In the case of clathrin, the energy of deformation probably also comes from coat assembly, but the fission step requires enzymatic activity from a GTPase, dynamin. Dynamin is recruited to coated pits and, under conditions that interfere with its GTPase activity, dynamin forms a collar or ring around the neck of the budding vesicle. Whether this protein acts as a mechanochemical transducer to generate fission (the 'boa constrictor', 'blue collar' or 'pinchase' model)[71–73], as a recruiter to attach other proteins that are directly responsible for the fission step (the 'rattlesnake' or 'white collar'

J DOMAIN
Approximately 73 amino-acid region found in DnaJ-like heat shock proteins, which catalytically activates proteins of the Hsc70 family.

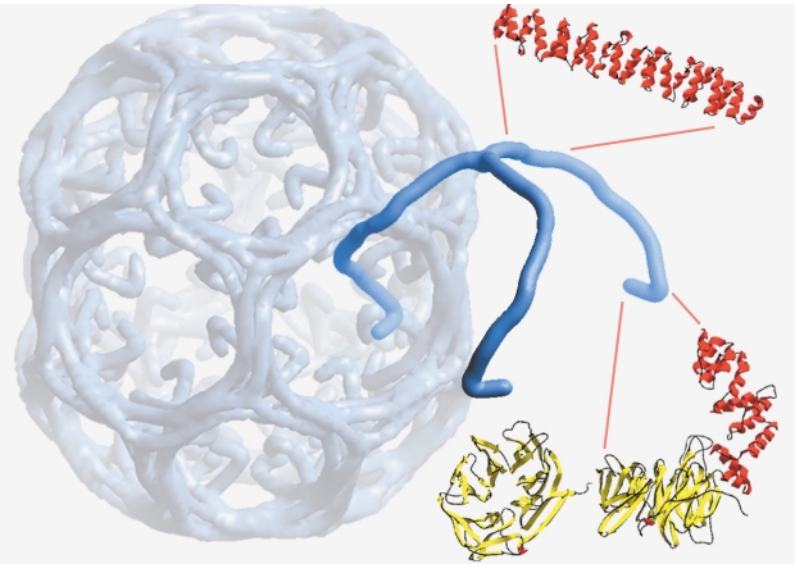

Figure 6 | **Clathrin coats, a collage of medium and high-resolution views.** The model of a clathrin cage at 21-Å resolution was obtained by electron microscopy[92]. The clathrin triskelion is a puckered and relatively rigid molecule[93]. The proximal[84] and distal[81] leg domains of the clathrin heavy chain have similar α-zigzag atomic structures, and the globular terminal domain of the clathrin heavy chain is a β-propeller[81].

model)[72,74,75] or as a combination of both is still a matter that needs to be resolved.

Lipid-modifying enzymes such as endophilin, synaptojanin and phospholipase D are also involved in vesicle formation[59,74,76–78]. Endophilin is an acyltransferase that interacts with dynamin and that generates lysophosphatidic acid by fatty acyl transfer from arachidonic acid or palmitic acid[74]. The current view is that this reaction produces a negative curvature at the neck of the vesicle, which is postulated to facilitate pinching-off of the membrane in the fission step[79,80]. Synaptojanin acts in the endocytic pathway to remove the 5-phosphate from phosphatidylinositol-4,5-bisphosphate, probably modulating the recruitment of phosphoinositide-binding proteins such as AP-2, β-arrestin and dynamin to the plasma membrane. Phospholipase D produces phosphatidic acid, modulating membrane curvature and increasing the concentration of the second messenger diacyl glycerol[79]. The relative importance of these different enzymatic activities is not yet clear but, once again, there is obviously a greater complexity in the clathrin pathway than in the COP pathways and greater potential for regulation.

Visualization at medium and high resolutions. Considerable progress has been made in the visualization of some elements of the clathrin coat using a combination of X-ray crystallography and electron microscopy[41,81–93]. This information has allowed us to understand the molecular basis of some of the interactions that are known to occur within the coat, including sorting and coat formation. Because of the size and complexity of many of the coat components, the first atomic views are of recombinant fragments from adaptors, amphiphysin, dynamin, epsin, Eps15 and clathrin[41,81–91].

SH3 DOMAINS
(Src homology region 3 domains.) Protein sequences of about 50 amino acids that recognize and bind sequences rich in proline.

The μ subunit of AP complexes is the portion of the adaptor that recognizes short peptides bearing YppØ sorting signals[94]. Detection of this interaction led to further experiments based on combinatorial approaches[95,96] that established rules of engagement between YppØ sorting signals and μ-subunits found in different APs. These observations were rapidly followed by the determination of the atomic structure of a fragment of the μ2 subunit of AP-2, either alone or in association with short peptides bearing the endocytic signals YQRL from TGN38 or YRAL from the EGF receptor[82].

For several reasons, these atomic models have had a large effect on our understanding of the mechanism of sorting. First, they show that the structure of μ2 does not change on interaction with peptides bearing sorting signals, arguing that this portion of the adaptor is relatively rigid. Second, they show that the sorting signals adopt an extended conformation on binding to μ2, and they therefore help to explain the basis of specificity in the recognition of sorting signals by different μ-subunits. Third, they will facilitate the implementation of mutagenesis studies by aiding in the design of μ-variants for *in vivo* and *in vitro* studies. Structures have also been determined of fragments corresponding to the globular carboxy-terminal 'ears' of the α- and β-adaptins of AP-2[83,87,91], the EH domain of Eps15 (REFS 85,86), the SH3 DOMAIN of amphiphysin 2 (REF. 90), part of the ENTH domain of epsin[89] and the pleckstrin-homology domain of dynamin[88]. As with μ2, these structures will probably facilitate the design and interpretation of functional experiments.

The structural studies of clathrin and its partners have had a clear and important effect on the way that we now think about coat formation, and provide a basis for understanding the mechanism of coat formation and the interaction between clathrin and adaptors. The crystal structures of two large fragments of the clathrin heavy chain, comprising about half of its total mass, are now known[81,84] (FIG. 6). One fragment contains the globular amino-terminal domain of the heavy chain linked to a segment, about 100 Å long, that joins this domain to the distal portion of the clathrin leg[81]. The atomic structure of the amino-terminal domain was shown to be a seven-bladed β-propeller[81] with a groove between blades 1 and 2 that accommodates the clathrin box[41], a short motif found in proteins that interact with clathrin (for example, β-arrestin and APs)[5,62]. The structure of the amino-terminal domain bound to a clathrin-box peptide is consistent with the inward orientation of the amino-terminal domains within a coat revealed by fitting the atomic structure of the amino-terminal domain and linker to the lower-resolution model of a clathrin coat obtained by electron microscopy[93].

The second crystallized fragment maps to the opposite end of the leg, and corresponds to a linear stretch of about 100 Å derived from the proximal leg, the region involved in the interaction with light chains[84]. Comparison of the two structures reveals that the extended segments are similar. They contain a polypeptide chain folded into an 'α-zigzag', a series of short, apposed α-helices that run back and forth,

roughly perpendicular to the overall direction of the leg. The parts of the leg not contained in the crystal structures are known to have a high α-helical content and they are also believed to contain α-zigzags. The α-zigzag is not a completely rigid fold, as shown by a modest variability of curvature in the linker segment seen in the crystals of the fragment containing the amino-terminal domain and the linker. The extent of this flexibility is sufficient to accommodate the changes required for the formation of pentagons and hexagons in the facets of the coat, particularly at the joints between the proximal and distal legs.

Electron microscopy studies of clathrin triskelions and coats have revealed how the legs interact within a surface lattice[92,93,97,98] (see online animation). This has led to a model that explains how a coat can form by the sequential addition of soluble clathrin molecules to the assembling lattice[93]. The model highlights the idea that the addition of triskelions can happen at various locations on the lattice but that rearrangement of elements in a preformed coat (from hexagons to pentagons or vice versa) is topologically impossible[36]. That is, a budding coated pit must form by the sequential incorporation of coat elements (clathrin, adaptors, and so on) and not by the direct transformation of a pre-existing flat array into a curved coat. Flat arrays[99], which abound at the plasma membrane and in the TGN, could act as dynamic reservoirs, similar to the relatively stable COPII reservoirs on ER membranes discussed above, disassembling at their margins to provide triskelions for *de novo* coat formation in the immediate vicinity.

Studies in live cells. Time-lapse fluorescence microscopy has been used to observe GFP-tagged clathrin in *Dictyostelium*[100] and mammalian[101] cells. In *Dictyostelium*, the endogenous clathrin heavy chain was replaced with heavy chain tagged with GFP at its carboxyl terminus. Bright spots appeared at the plasma membrane, the perinuclear region and the cytosol that were highly mobile and seemed to move in synchrony, like a swirl following the internal motions of the cell. The highly mobile spots persisted for 30 seconds or less and their disappearance could reflect uncoating or movement away from the plane of focus. Thus, the results from this experiment do not resolve the question of whether the observed dynamic behaviour reflects the budding cycle of a clathrin-coated vesicle.

In mammalian cells, transient expression of GFP-tagged clathrin light chain A resulted in bright spots associated with the plasma membrane and bright patches located in the perinuclear region[101]. The bright spots were stable most often, very much as described above for Sec13p–GFP. Occasionally, it was possible to observe smaller and weaker spots emanating from locations at the plasma membrane close to the brighter ones. The proposed interpretation of the data was that all the spots represent various stages in the process of budding of coated pits and formation of coated vesicles. A word of caution is needed, however. The expected level of replacement of the endogenous light chains by LCa–GFP by transient expression is limited. It is possible that the bright, stable spots represented the large, flat, hexagonal flat arrays of clathrin acting as potential reservoirs for coated-vesicle formation, rather than vesicles themselves. The smaller coated pits and vesicles (with <60–100 triskelions in the coat) would be harder to detect and, in general, the intensity of the fluorescence signal would probably have been close to the detection limits (5–10 GFP molecules per location).

Where are we heading and what can we expect?

Current research is attempting to define the remaining components that participate in the COPI, COPII and clathrin pathways. Structural and functional comparisons between the elements of each of these systems have proved to be very useful. Efforts to understand other routes of traffic are just starting to bear fruit, using a combination of genetics and biochemistry. Soon, there should be a better understanding of how molecules move between all membrane-bound intracellular compartments, whether by coated vesicles or by vesiculo–tubular structures. With the complete list in hand, it should be possible, for any given pathway, to do the appropriate biochemical characterization and to obtain a mechanistic description, at atomic resolution, of the interactions that regulate the traffic.

These pathways are strikingly complex and have a large number of components and an even larger number of interactions and regulating steps that are used to control proper membrane flow. Until recently, it was thought that relatively simple genetic manipulations such as gene disruptions would provide direct clues to the function of any given protein component. However, the multicomponent character of the coated-vesicle-based pathways frequently allows compensation, sometimes to the extent that only a weak cellular phenotype is manifest, even in a multiple knockout. In some cases, it will be possible to obtain important and useful information by overexpressing defective proteins or molecules modified to a fixed state of their normal cycle. However, other forms and techniques for selective dissection are needed, including the development of improved methods for visualizing small vesicles and molecules as they form and move inside live cells, and the discovery and use of small molecules that can enter cells and act acutely and specifically on given steps of a selected pathway.

Links

DATABASE LINKS Sec23p | Sec24p | Sec13p | Sec31p | Sar1p | Sec12p | Emp24 | COPI coatamer | KDEL receptor | ARF1 | ARNO3 | ARFGAP | LDL receptor | clathrin | AP-1 | AP-2 | arrestins | synaptotagmin | amphiphysin | epsin | synaptojanin | Eps15 | dynamin | Hsc70 | endophilin | phospholipase D | EH domain | ENTH domain | Pleckstrin homology domain

FURTHER READING For a more detailed description of clathrin's binding partners, see Slepnev, I. V. & Camilli, D. P. Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nature Rev. Neurosci.* **1**, 161–172 (2000). [Contents page]

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