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Clathrin and adaptors

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Abstract

Clathrin and adaptors are components of clathrin-coated pits and vesicles. The AP-1 adaptor complex is associated with clathrin-coated vesicles budding from the TGN, while the AP-2 adaptor complex is associated with clathrin-coated vesicles budding from the plasma membrane. The clathrin forms a polyhedral lattice and is believed to be the driving force behind membrane invagination leading to vesicle budding. The adaptors attach the clathrin to the membrane and also interact with the cytoplasmic domains of selected transmembrane proteins, causing these proteins to become concentrated in clathrin-coated vesicles. Clathrin-coated vesicles budding from the TGN have been implicated in the sorting of newly synthesised lysosomal enzymes, while clathrin-coated vesicles budding from the plasma membrane facilitate the receptor-mediated endocytosis of ligands, such as low density lipoproteins and transferrin. A novel adaptor-related complex, AP-3, has recently been identified, which is recruited onto membranes of the TGN and a more peripheral compartment but does not appear to be associated with clathrin. Genetic studies indicate that AP-3 plays a role in the sorting of proteins to lysosomes and lysosome-related organelles.

Keywords: Clathrin; Adaptin; Adaptor; AP-1; AP-2; AP-3; *trans*-Golgi network

1. Historical perspective

Coated pits and vesicles were first described 34 years ago by Roth and Porter in their studies on yolk protein uptake in mosquito oocytes. They found that during the time that the cells were actively taking up yolk, their surfaces became highly specialised, with numerous invaginations and vesicles just under the plasma membrane. Both the invaginations and the vesicles were covered with bristle-like projections on their cytoplasmic sides, forming a ~20 nm coat. Deeper in the cell, they observed vesicles of a similar diameter, but without the coat. Based on these observations, Roth and Porter proposed that when yolk proteins bind to the oocyte surface, the membrane invaginates to form bristle-coated pits. These pits pinch off to form coated vesicles which carry the adsorbed protein into the cell. The bristle coat is then lost, and the vesicles fuse to form a storage granule. From these studies they postulated that coated pits were responsible for taking up extracellular material, not just in oocytes but in other types of cells as well. With remarkable foresight they speculated that the formation of coated pits and vesicles was likely to be transient in nature, that the bristle coat might play a mechanical role in the formation of the pits and vesicles, and that it might also provide some selectivity for what is adsorbed [1].

Three years later a second population of coated vesicles was described by Friend and Farquhar in

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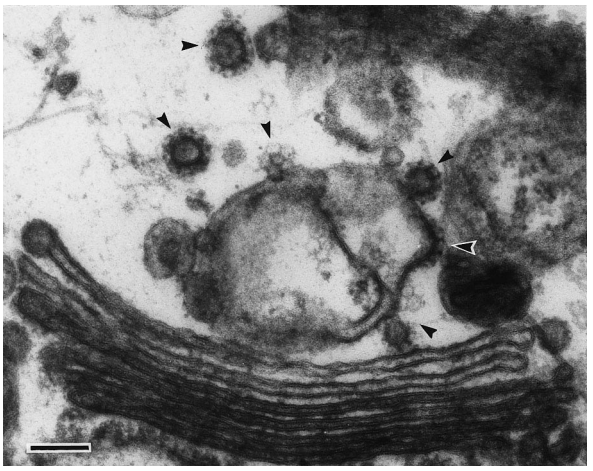

Fig. 1. Clathrin-coated vesicles budding from the TGN. NRK cells were permeabilized by freezing and thawing, then incubated with pig brain cytosol plus GTPγS to stimulate the recruitment of AP-1 and clathrin onto the TGN. Clathrin-coated buds are indicated, including some sectioned tangentially where the lattice is apparent. Scale bar: 100 nm.

the Golgi region of the cell [2] (Fig. 1). They showed that the Golgi coated vesicles had a similar morphology to the bristle-coated vesicles that Roth and Porter had described (although with the benefit of hindsight it is now clear that some of the structures they were observing were clathrin-coated vesicles while others were COPI-coated vesicles), and they proposed, based on cytochemical staining, that at least one function of the Golgi coated vesicles was to transport hydrolytic enzymes to lysosomes. Another major advance was the isolation of coated vesicles from pig brain in 1969 by Kaneseki and Kadota [3]. These authors were the first to show that although the coat appears bristle-like in thin sections, in negatively stained preparations it can be seen to consist of a lattice-like polyhedral network covering the vesicle, which they called 'the vesicle in a basket'. However, it was not until the purification of these vesicles to near homogeneity by Pearse in 1975 that the study of the protein composition of the coat became possible. The major protein band in purified coated vesicles was found to be a protein of 180 kDa, named clathrin [4]. Clathrin was subsequently purified from coated vesicle extracts by gel filtration and shown to be the only component necessary in vitro to reform lattice-like structures resembling vesicle coats [5,6]. Studies on purified clathrin also showed that in its native form, clathrin consists not only of the 180 kDa protein, now called clathrin heavy chain, but also two other proteins with apparent molecular weights (in brain) of 33 and 36 kDa, the clathrin light chains, and that the basic clathrin assembly unit is the triskelion, consisting of three copies of clathrin heavy chain and three copies of clathrin light chain [7,8].

At about the same time that clathrin was being identified and characterised, Brown and Goldstein and their collaborators were carrying out studies that definitively established the function of clathrin-coated vesicles. Brown and Goldstein's earlier work had shown that low density lipoproteins (LDL) are internalised very efficiently by receptor-mediated endocytosis, many times faster than the rate predicted from measurements of bulk endocytosis [9,10]. When they examined cells in the electron microscope that had been incubated with ferritin-labelled LDL at 4°C, they found that ~70% of the label was in clathrin-coated pits, even though clathrin-coated pits only represented ~2% of the total surface area of the plasma membrane. If the cells were warmed to 37°C before fixation, they found that the LDL moved rapidly from coated pits to coated vesicles to uncoated vesicles, just as Roth and Porter had predicted [11]. Additional evidence for the role of clathrin-coated vesicles in selective endocytosis came from their studies on cells from J.D., a patient with the genetic disorder familial hypercholesterolaemia (FH). Unlike most patients with FH, J.D.'s cells could bind LDL, but the internalisation rate was very slow. When they examined the bound LDL in the electron microscope, they found that it was randomly distributed on the plasma membrane rather than concentrated in clathrin-coated pits [12]. From these studies they proposed that the LDL receptor was a transmembrane protein with a ligand binding site on the extracellular side of the membrane and an internalisation site on the cytoplasmic side of the membrane, and that it was this internalisation site that was defective in the J.D. cells. The sequencing of both the wild-type LDL receptor and the J.D. receptor some ten years later confirmed their prediction, and showed that a crucial tyrosine residue in the cytoplasmic tail of the wild type receptor was mutated to a cysteine in the J.D. receptor [13].

Soon after Brown and Goldstein's seminal work on the LDL receptor, a number of other plasma

membrane proteins were shown to be concentrated in clathrin-coated pits and vesicles, including the transferrin receptor [14], the asialoglycoprotein receptor [15], and the epidermal growth factor receptor [16,17]. Identifying proteins that were concentrated in clathrin-coated pits and vesicles in the Golgi region of the cell proved to be more difficult. However, it was at about this time that the mannose-6-phosphate (M6P) receptor for lysosomal enzymes was identified [18], and biochemical and ultrastructural studies showed that lysosomal enzymes bound to the M6P receptor on the cell surface were rapidly internalised into clathrin-coated vesicles [19,20]. Since there was evidence to suggest that major route taken by newly synthesised lysosomal enzymes was from the Golgi directly to the lysosome, bypassing the plasma membrane [21,22], these observations indicated that M6P receptors might also be concentrated in Golgi-derived clathrin-coated vesicles.

Although Brown and Goldstein originally suggested that an internalisation signal in the cytoplasmic tail of the LDL receptor might bind directly to clathrin, subsequent studies revealed that clathrin was not the only component of the coat, nor the most likely candidate for a receptor-binding protein. A study by Keen and co-workers in 1979 [5] showed that coats could be extracted from clathrin-coated vesicles with 0.5 M Tris-HCl, after which gel filtration was used to separate the clathrin (both heavy and light chains) from a fraction containing proteins with a molecular weight of ~100 kDa. When dialysed back into a favourable buffer, the clathrin was able to reassemble, but reassembly occurred under less stringent conditions if the 100 kDa-containing fractions were added as well. These 100 kDa proteins were named assembly polypeptides, or APs [5]. Subsequent studies, both biochemical and ultrastructural, indicated that the APs were positioned between the clathrin lattice and the vesicle membrane [23–25]. The term adaptor was first proposed in 1981 by Pearse and Bretscher as a name for the putative factor that would link proteins like the LDL receptor to clathrin [26], and the positioning of the APs in the clathrin-coated vesicles suggested that adaptors and APs might be one and the same.

Further characterisation of the APs demonstrated that they are hetetromeric protein complexes containing proteins of ~50 kDa and ~20 kDa as well as two proteins of ~100 kDa, and that there are two distinct AP complexes which can be separated by hydroxylapatite chromatography, AP-1 and AP-2 (also sometimes called HA-I and HA-II) [27,28]. Immunofluorescence using antibodies against the 100 kDa subunits, or adaptins, revealed that AP-1 is associated with the Golgi apparatus (specifically the *trans*-Golgi network) and AP-2 is associated with the plasma membrane [29,30]. A number of different types of studies have been carried out over the past ten years which indicate that the APs do indeed bind to internalisation or sorting signals in the cytoplasmic domains of proteins that end up as cargo in clathrin-coated vesicles. Approaches have included binding adaptors to peptides or fusion proteins on affinity matrices, co-immunoprecipitation of adaptors and receptors, and (more recently) making use of the yeast two-hybrid system and surface plasmon resonance [31–33].

Recently, a third AP complex has been discovered, which unlike AP-1 and AP-2, is not associated with clathrin. In other respects, however, the new complex, AP-3, closely resembles AP-1 and AP-2. It is a heterotetramer made out of subunits related to those in AP-1 and AP-2, it is recruited onto membranes in a similar fashion, and it appears to bind to a similar repertoire of sorting signals [34–36]. Whether AP-3 associates with another scaffolding protein which might be taking the place of clathrin remains to be seen, but electron microscopy indicates that if such a protein exists, it must have quite a different structure from clathrin [34].

2. Clathrin

2.1. Genes and proteins

Clathrin heavy chain is ubiquitously expressed in all eukaryotic cells. Southern blot analysis originally indicated that there was a single clathrin heavy chain gene in mammals [37], but recently a protein that shares 83% amino acid identity with clathrin heavy chain was identified [38]. The high degree of homology suggests that this protein may be functionally equivalent to the original clathrin heavy chain, although its expression pattern is different, being mainly expressed in skeletal muscle. The conserved

properties of the clathrin heavy chain gene have been used to isolate homologues from the budding yeast *Saccharomyces cerevisiae* [39] and the cellular slime mold *Dictyostelium discoideum* [40]. Yeast clathrin heavy chain shares 50% amino acid identity with rat clathrin heavy chain and 45% amino acid identity with *Dictyostelium* clathrin heavy chain.

In contrast, the clathrin light chains are more polymorphic. Mammals express two clathrin light chains, LCa and LCb, which are the products of distinct genes, sharing 60% amino acid identity [41]. Between mammalian species the light chains are highly conserved, with 95–98% amino acid identity. The differing abilities of LCa and LCb to stimulate uncoating ATPase activity (see below) [42] and to be phosphorylated [43], and the unusually high ratio of LCb to LCa in cells with a regulated secretory pathway [44], suggest that there are functional differences between the two light chains. However, both light chains appear to bind to the heavy chain in a similar manner and they are distributed randomly on clathrin triskelions [45,46]. In the brain, there are two additional forms of LCa and one of LCb produced by alternative splicing, and it has been proposed that these alternatively spliced isoforms interact with brain-specific factors [41]. Interestingly, despite the similarity of the mammalian and yeast clathrin heavy chains, yeast has only a single clathrin light chain gene, and the yeast protein shares only 16% amino acid identity with mammalian LCa and 18% amino acid identity with mammalian LCb [47].

2.2. Function of the heavy and light chains

While clathrin heavy chains clearly provide the structural framework for the polyhedral lattice, the role of the clathrin light chains remains unclear and elusive. On the one hand, clathrin heavy chain can assemble in the absence of light chains [46], and the presence of light chains does not affect the stability of the coat [48]. However, deleting the clathrin light chain gene in yeast has the same phenotype as deleting the clathrin heavy chain gene [47], indicating that the light chain is essential for clathrin function, and more recent studies on yeast have shown that clathrin heavy chain trimerisation is perturbed by the absence of light chains [49]. In addition, clathrin light chains have been shown to contain a calcium binding site [50], and since, at least in vitro, clathrin assembly can be modulated by calcium, this has led to the proposal that the light chains may play a regulatory role in clathrin assembly. The light chains have also been shown to stimulate the phosphorylation of the one of the AP-2 subunits [51], and LCb can be phosphorylated by casein kinase type II [43,52], although at present the role of phosphorylation in coat assembly is unclear. It has also been suggested that the clathrin light chains may play a role in coat disassembly, since the uncoating ATPase Hsc70 (heat shock cognate 70, see below) is dependent on the presence of clathrin light chains, at least in vitro [48].

2.3. Clathrin assembly

The property of clathrin to self-assemble in vitro has been demonstrated for mammalian [5–7], plant [53], and yeast [54] clathrin. Although yeast clathrin lattices appear to be less stable, the ability of clathrin to assemble into a cage-like structure demonstrates that it is ideally designed to form the scaffold which, when attached to a membrane, would cause that membrane to deform into a budding vesicle.

The basic assembly unit of the clathrin lattice is the triskelion [7]. Morphologically, there are three distinct regions in each 'leg' of the triskelion. The 17 nm long proximal segment (next to the vertex) is divided by a bend from the 25 nm long distal segment, and this is followed by a globular domain at the far end of the vertex [55]. The globular domain is composed of the N-terminal third of clathrin heavy chain, connected to the rest of the triskelion by a short flexible linker region which allows this domain to project inwards [24,25,37,56]. Estimates of the triskelion molecular weight by equilibrium sedimentation and electron microscopy (630×10^3) first suggested that each triskelion was composed of three heavy chains and three light chains, [7]. This was confirmed by crosslinking studies, which further suggested that each heavy chain was in close contact with a light chain [8]. Using electron microscopy of negatively stained preparations, Crowther and Pearse were able to elucidate the way in which the clathrin triskelions were packaged to form the latticework seen around the coated pits. They proposed that each triskelion leg spans two edges of a coated vesicle

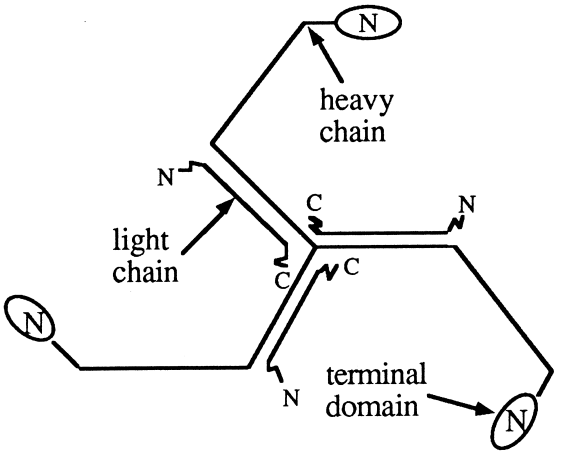

Fig. 2. Model of a clathrin triskelion. Drawing by Dr. Catriona Ball.

lattice, and every lattice edge includes contributions from four triskelions [6] (Figs. 2 and 3).

Immunolocalisation studies indicate that the clathrin light chains are bound along the proximal leg of the clathrin triskelion [57,58]. The binding site on clathrin light chain for clathrin heavy chain has been mapped using monoclonal antibodies [58]. This region of the light chain contains a series of strongly conserved heptad repeats that are characteristic of α-helical coiled coils [41,59], and it has been suggested that the heptad repeats of the light chains interact with a region of the clathrin heavy chain that also has a weak pattern of heptad repeats [37]. More recently, a truncated form of clathrin heavy chain has been expressed in bacteria, consisting of the C-terminal third of the protein, which is able to trimerize, to bind light chains, and even to self-assemble to some extent, although the lattices formed by the truncated heavy chain are relatively disorganised [60].

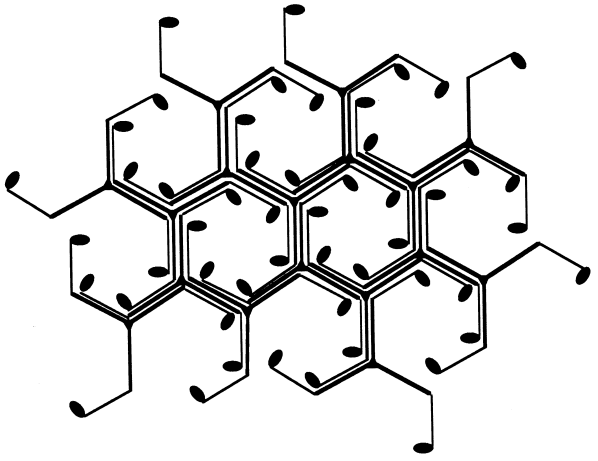

Fig. 3. Assembly of clathrin triskelions into a lattice. Drawing by Dr. Catriona Ball.

2.4. Clathrin lattice to coated vesicle

As a problem in geometry, the structure of the clathrin lattice and its transformation into a vesicle coat is straightforward. Early studies on isolated clathrin-coated vesicles demonstrated that the coat consists of 12 pentagons and a variable number of hexagons [3,6], 12 pentagons being the number required to make a closed structure. More dramatic images of the organisation of the coat were produced by Heuser in 1980, using a novel quick-freeze, deep-etch, rotary shadowing method. This technique was applied both to isolated coated vesicles and to ripped-open cells, where striking views of coated pits forming on the cytoplasmic surface of the plasma membrane were obtained. These structures ranged from flat lattices made up exclusively of hexagons to more curved lattices, consisting of pentagons as well as hexagons (Fig. 4). Thus, it was proposed that the clathrin coat is laid down initially as a flat network of hexagons, some of which are then converted into pentagons, thus driving the curvature process [61]. The ability of clathrin to self-assemble in vitro into structures containing pentagons as well as hexagons supports the view that it is the clathrin that makes the membrane change its shape. However, an alternative view has been proposed which so far cannot be ruled out: that the introduction of pentagons into the lattice is a consequence of membrane curvature (perhaps induced by changes in the lipid bilayer) rather than the driving force behind it [62].

There is an additional problem, which is how the clathrin lattice rearranges itself to convert some of the hexagons to pentagons. According to the Crowther and Pearse model, each polyhedron edge contains two proximal segments and two distal segments, contributed by four different triskelions. These interactions must all be broken and new interactions must be set up if a hexagon is to be replaced by a pentagon. Although in vitro systems have been developed which are beginning to dissect the requirements for individual steps in the formation of a coated vesicle [63], the actual mechanism that results

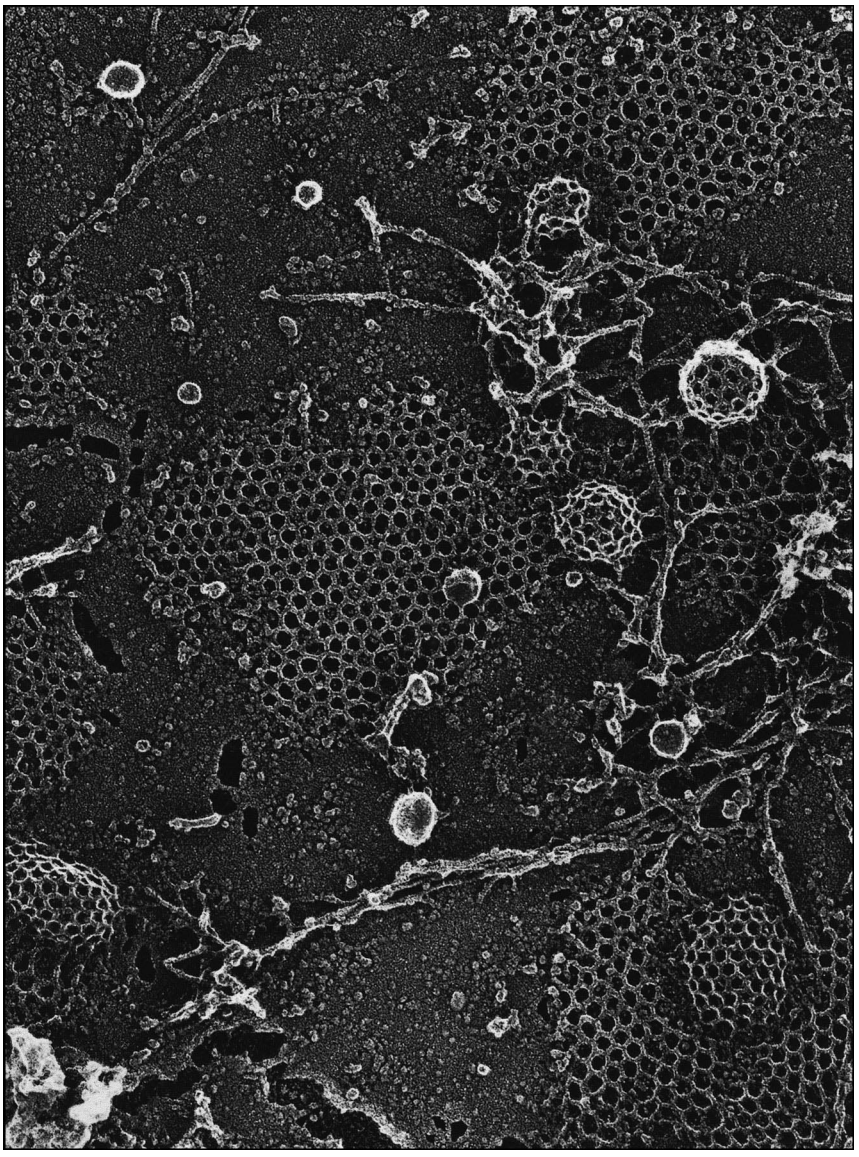

Fig. 4. Cytoplasmic face of the plasma membrane, prepared using a rapid freeze, deep etch, rotary shadowing method. Note the flat clathrin lattices, consisting mainly of hexagons, and the rounder, deeply invaginated clathrin-coated pits, consisting of pentagons as well as hexagons. Electron micrograph courtesy of Dr. John Heuser.

in the transformation of hexagons into pentagons is still unknown.

3. Adaptors

3.1. Genes and proteins

Both the TGN-associated AP-1 adaptor complex and the plasma membrane-associated AP-2 adaptor complex consist of four subunits: a β subunit, a μ (medium chain) subunit, an σ (small chain) subunit, and a more divergent subunit, γ in the case of AP-1 and α in the case of AP-2. The β, γ, and α subunits (the adaptins) are all ∼100 kDa, the μ subunits are ∼50 kDa, and the σ subunits are ∼20 kDa. The four subunits are closely associated with each other and cannot be dissociated without the use of harsh denaturants. Cross-linking, gel filtration, and ultracentrifugation studies all indicate that each subunit is present at one copy per adaptor complex (Fig. 5) [27,28,30,64].

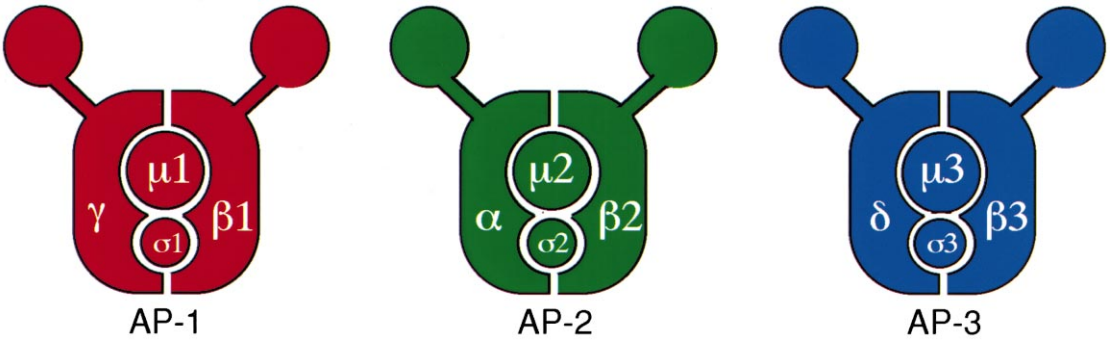

Fig. 5. Models of the AP-1, AP-2, and AP-3 complexes.

Although all the adaptor components are highly conserved from one mammalian species to another, this is particularly true for the β subunit of the AP-2 complex (β2), which is 100% identical between human and rat [65]. Like the clathrin light chains, β2 is alternatively spliced in neurones, with an additional 14 amino acids in its hinge domain (see below). β2 is 84% identical to the β1 subunit of the AP-1 complex [65,66]. In mammalian cells, there are two isoforms of α-adaptin encoded by different genes, named (for historical reasons) αA and αC, which are 80% identical [67]. It is not known if αA and αC are functionally equivalent, but they have been shown to be co-localised in the same plasma membrane coated pits. However, the relative amount of each isoform varies in different tissues, which may point to a specialised role [68]. αA is also alternatively spliced in neurones, with an extra 22 amino acids in its hinge domain. So far only a single isoform of γ-adaptin has been characterised, but Southern blotting indicates that there may be a second gene [69], and a second closely related sequence(s) can be found in the EST database. Sequence comparison has shown that there is very limited homology between α-adaptin and β-adaptin [65,67] and between γ-adaptin and β-adaptin [69,70]. However, α- and γ-adaptin exhibit higher homology, having ~25% sequence identity [69].

The medium chains (μ1 and μ2) and the small chains (σ1 and σ2) both share ~40% amino acid identity with each other [71–73]. Sequencing has also revealed limited, but significant, homology between the μ and σ subunits [74]. There is at least one additional isoform of μ1 and one of σ1, which can be found in the EST database, although so far it is not known whether these are functionally distinct from the known isoforms.

Homologues of the various adaptor subunits have been cloned from nematode worms [75], plants [76,77], *Dictyostelium* [78], *Drosophila* [79,80], and yeast [72,73]. In yeast, the products of the genes *APL4*, *APL2*, *APM1*, and *APS1* encode proteins that appear genetically and biochemically to be associated with clathrin and the equivalent of the AP-1 γ, β1, μ1, and σ1 subunits, respectively [81–83]. It has been proposed that the products of the genes *APL3*, *APL1*, *APM4*, and *APS2* may encode the equivalent of AP-2 in yeast [84], but there is as yet no functional data to support this. An AP-3 complex has also been identified in yeast (see below). However, it has yet to be resolved why there are three α/γ/δ-type proteins, three β-type proteins, and three σ-type proteins in yeast, and yet four μ-type proteins.

3.2. Structure of adaptor complexes

The structure of the AP-2 adaptor complex has been studied by rotary shadowing [85], and the isolated adaptors were shown to consist of a brick-like core or 'head' flanked by two symmetrically placed appendages or 'ears'. The ears are attached to the head by a ~6 nm stalk, thought to act as a flexible hinge, since the ears protrude at different angles in different specimens. Removal of the ears by limited proteolysis [86] has shown that they correspond to the C-terminal domains of α- and β2-adaptin [66]. The adaptor core or head, which is left behind after proteolysis, contains the N-terminal domains of α- and β2-adaptin, together with μ2 and σ2 [86]. The hinge domain, which is cleaved by the protease, is

very hydrophilic and is highly enriched in the amino acids proline, glycine, and alanine, similar to hinge regions in other proteins (see [87]). Proteolytic digestion of the AP-1 complex results in similar cleavage of the C-terminal domains of the two adaptins, suggesting a similar organisation [88] (Fig. 5).

Despite our knowledge of the structural organisation of the adaptors, at present little is known about the biosynthetic assembly of the adaptor complex and the interactions that take place between the various subunits as the complex forms. However, adaptor subunit interactions have been studied using the yeast two-hybrid system. These studies have shown that the α and γ subunits interact with both the β and the σ subunits, and that the β subunits also interact with the μ subunits [89]. Information about interactions between adaptor subunits has also come from experiments in which chimeras were constructed between α- and γ-adaptin and then immunoprecipitated under non-denaturing conditions from transfected cells to determine the composition of the complexes assembled from the various chimeras. From these studies, it has become clear that the μ subunits must also bind either to the α/γ subunits and/or to the σ subunits, indicating that some interactions occur which are not detected by the two-hybrid system [89].

Cryo-electron microscopy has been carried out on coats assembled in vitro either from clathrin alone or from clathrin plus adaptors, to map the position of the adaptors in the coat. When the two types of structures were compared, the coats assembled from clathrin plus adaptors were found to contain an additional inner layer, close to where the membrane would be. Coats assembled from clathrin alone and then treated with protease to remove the globular N-terminal domain were also examined, and projections pointing inwards, towards the adaptor layer, were found to be missing. Thus, from these studies it was concluded that the N-terminal domain of clathrin interacts directly with adaptors, which in turn interact with the membrane [24,25]. However, previous studies had shown that triskelions lacking the N-terminal globular domains are able to bind adaptors with the same affinity as intact triskelions [90]. This discrepancy was resolved when it was found that clathrin contains at least two distinct adaptor binding sites, one of which is only present in assembled clathrin coats [91,92]. Thus, it was proposed that adaptors may bind initially to the clathrin leg, but in the assembled coats only the interaction with the N-terminal domain is necessary.

3.3. Function of adaptor complexes

The first function attributed to adaptors was a role in coat assembly. Although clathrin triskelions are able to reassemble into cages in vitro in the absence of adaptors [5–7], the sizes of the cages vary considerably, and the reaction occurs only under non-physiological conditions. The addition of adaptors to the assembly mixture allows coats of a uniform size to assemble under physiological conditions [93].

It has also been proposed that adaptors mediate the binding of clathrin to the membrane, based in part on structural studies which indicate that adaptors are positioned between the clathrin lattice and the vesicle membrane [24,25]. Additional support for this proposal has come from biochemical experiments, which have shown that when clathrin is removed from coated vesicles by mild chemical treatment [23,94] or by incubation with the uncoating ATPase Hsc70 [95], the adaptors are still retained on the membrane. If such clathrin-stripped membranes are then treated with elastase to cleave the adaptors, the membranes are no longer able to rebind clathrin [23,90,96]. Further evidence indicating that adaptors attach clathrin to the membrane comes from experiments in which the AP-2 complex was 'mistargeted' to an endosomal compartment by the addition of GTPγS, and clathrin was found to 'follow' the AP-2 complex to this compartment (see below).

There is also evidence that adaptors bind to the cytoplasmic domains of selected transmembrane proteins (usually receptors for a ligand on the other side of the membrane), and enable them to become concentrated in the coated vesicle. The first study along these lines was one in which the M6P receptor, which is concentrated in clathrin-coated vesicles both at the plasma membrane and at the TGN, was shown to form aggregates with AP-2 when incubated under detergent-free conditions [97]. In subsequent experiments, the interaction of receptors with adaptors was investigated by constructing affinity columns containing the cytoplasmic tails of either the LDL receptor

or the M6P receptor [31,98], over which purified iodinated adaptors were passed. The AP-2 complex was found to bind to both the LDL receptor tail matrix and the M6P receptor tail matrix, while the AP-1 complex only bound to the M6P receptor tail matrix. Together, these in vitro binding assays indicate that the adaptors interact with trafficking receptor tails both at the plasma membrane and at the TGN. In vivo support for the role of adaptors in receptor binding has come from experiments in which the EGF receptor was immunoprecipitated and AP-2 was shown to come down as well [32,99]. More recent studies have narrowed down both the sorting signals that enable certain proteins to bind to adaptors and the adaptor subunits that mediate the binding.

3.4. Function of adaptor subunits

Although the adaptor subunits are tightly associated with each other, a number of techniques have been used to investigate the functions of the individual proteins. There is considerable evidence implicating the β-adaptins in clathrin binding. The high degree of sequence conservation between β1 and β2 [66], indicated by the similarity of their peptide maps even before the two proteins were cloned [30], made them likely candidates for this role. In an in vitro binding assay, purified β2-adaptin was shown to interact with clathrin with a stoichiometry of nearly one to one, and β2 was also found to compete with the entire AP-2 complex for binding to clathrin, suggesting that the β subunit contains the major clathrin binding site [100]. More recently, experiments have been carried out using recombinant β1 and β2, and both proteins on their own were found to be capable not only of interacting with clathrin, but also of promoting clathrin assembly [101]. In a subsequent study, a clathrin binding determinant on β2 was localised to the hinge region of the protein [102], and presumably the corresponding region of β1 mediates clathrin binding in the AP-1 complex.

It has also been suggested that the β subunits may be involved in the recognition of receptor tails, based on a study in which the purified asialoglycoprotein receptor was found to bind to the β2 band on Western blots of clathrin-coated vesicles [103] and one in which β2 was shown to bind a fusion protein containing the cytoplasmic tail of the EGF receptor [104]. However, the most compelling evidence points to a role for the adaptor medium chains in receptor binding and sorting signal recognition. At least three distinct types of sorting signals have been identified, all of which can promote efficient endocytosis as well as influence intracellular trafficking: the YXXØ motif (where Ø is a bulky hydrophobic amino acid), the di-leucine motif, and the FXNPXY motif, of which the best characterised is the YXXØ motif. In 1995, Ohno et al. [33] screened a yeast two-hybrid library using a triple repeat of such a motif (SDYQRL) from the cytoplasmic tail of TGN38 to search for interacting proteins. Out of over two million clones, two specifically bound to the SDYQRL motif, and both were found to encode the μ2 subunit of the AP-2 complex. Alanine substitutions showed that the two residues essential for this interaction were the tyrosine and the leucine, and these are also the two residues that are essential for the efficient endocytosis of TGN38. Ohno et al. went on to show that YQRL-containing constructs also bind to the μ1 subunit of the AP-1 complex, although more weakly. In contrast, the YXXØ motif of the transferrin receptor, YTRF, only interacted with μ2 in the two-hybrid system and not μ1, suggesting a mechanism for including the transferrin receptor (and presumably other proteins as well) in clathrin-coated vesicles budding from the plasma membrane, but not in clathrin-coated vesicles budding from the TGN [33]. There are as yet no candidates for adaptor subunits that might recognize the di-leucine signals and the FXNPXY signal. However, in vivo studies show that overexpressing YXXØ-containing proteins retards the internalisation of other YXXØ-containing proteins but not di-leucine-containing proteins, while overexpressing di-leucine-containing proteins retards the internalisation of other di-leucine-containing proteins but not YXXØ-containing proteins, indicating that even if both motifs bind to the AP-2 complex, they must bind to different sites [105].

The function of the α- and γ-adaptins has not been fully clarified, although they have been implicated in a number of protein–protein interactions. Since purified α-adaptin has been shown to co-immunoprecipitate with soluble clathrin triskelions, a role in clathrin binding has been suggested [106], although

the current consensus is that the β subunits play the major role in clathrin binding. Another role that has been attributed to α- and γ-adaptin is in targeting the two adaptor complexes onto the correct membrane. The C-terminal ear domains of α and γ show the least homology with each other, and initially it was predicted that these domains might control the specificity of adaptor binding [107]. However, when ear-swap chimeras were constructed between α- and γ-adaptin, the ear was found to have only a minor effect on adaptor localisation [108]. Similarly, proteolytic removal of the α- and γ-adaptin ears did not affect their ability to be recruited onto the appropriate membrane compartment in vitro [109,110]. In contrast, when chimeras were made within the N-terminal domains of α and γ, amino acids ∼130–330 were found to be crucial in determining where the adaptors were localised. When the adaptor complexes assembled from these chimeras were investigated, it was found that this same stretch of sequence determined whether a particular construct co-assembled with μ1 and σ1, or with μ2 and σ2 [89]. Thus, one or the other or both of these other subunits may also be involved in targeting. The α and γ subunits have also been shown to bind, either directly or indirectly, to other molecules involved in the clathrin-coated vesicle cycle, although at present the precise function of most of these other molecules is still unknown. The α ear binds to Eps15 [111] and to amphiphysin, which in turn binds to dynamin [112], while the γ ear binds to γ-synergin [113].

There is very little information concerning the role of the σ subunits, although their high degree of sequence conservation suggest an important function or functions. One such function, based on the correlation between the presence of a particular σ (and μ) subunit and adaptor localisation, may be to help direct the complex to the appropriate membrane [89].

3.5. Binding of adaptors to membranes

The targeting of adaptors to membranes is very precise. AP-2 binds specifically to the plasma membrane, while AP-1 binds to the TGN and to TGN-related compartments: immature (but not mature) secretory granules in cells with a regulated secretory pathway [114], and to some extent to an early endosomal compartment [115]. The way in which adaptors are targeted to specific membranes is still poorly understood. There is evidence that the presence of proteins that will end up as cargo in clathrin-coated vesicles at least contributes to adaptor recruitment. Thus, in cells that lack M6P receptors, the total amount of AP-1 binding to the TGN is reduced by ∼25%, while cells overexpressing M6P receptors show increased AP-1 binding [115]. Similarly, in cells overexpressing the transferrin receptor, the amount of clathrin lattice associated with the plasma membrane has been found to increase [116]. However, these proteins cannot by themselves provide the specificity for adaptor recruitment, since they may be more concentrated in compartments that do not recruit adaptors. For instance, in many cells, at steady state, the majority of the M6P receptors are in late endosomes, while the majority of the transferrin receptors are in early endosomes.

Various studies have suggested the existence of adaptor receptors or docking sites that could provide the specificity of binding. In 1988, Virshup and Bennett identified a high affinity AP-2 binding site on brain membranes that had been stripped of peripheral proteins [64]. Similarly, clathrin and adaptor reassembly onto stripped plasma membranes was shown to be a saturable process [96], suggesting that there is a limited number of membrane docking sites. These sites were shown to be sensitive to elastase, and they were not removed by high salt or carbonate treatment [117]. Together, these results suggest that there is an integral membrane protein or proteins that acts as a specific adaptor receptor. Peripheral membrane proteins also appear to contribute to adaptor binding. Studies on the binding of AP-1 to immature secretory granule membranes in regulated secretory cells showed that if the membranes were stripped with high concentrations of Tris or KCl, AP-1 was still able to be recruited from whole cytosol. However, when partially purified AP-1 was added, it could be recruited onto untreated membranes but not onto stripped membranes, suggesting that there are peripheral membrane proteins required for recruitment which can be supplied by the cytosol [114].

A putative candidate for the AP-2 receptor has been proposed by Zhang and co-workers, who showed that recombinant synaptotagmin attached

to agarose beads was able to bind with high affinity to AP-2 [118], although it is possible that the interaction that was observed may reflect the ability of synaptotagmin to be included in clathrin-coated vesicles as cargo, since it has not been demonstrated that synaptotagmin is actually required for AP-2 recruitment onto the plasma membrane. A number of other attempts have been made to identify integral membrane proteins that might act as docking sites. Mallet and Brodsky described proteins of 83 and 52 kDa present in detergent extracts of cell membranes which bound affinity columns of AP-1, but not AP-2 [119]. Seaman and co-workers [120] introduced a chemical crosslinker to permeabilised cells that had been allowed to recruit adaptors in vitro and found that proteins with molecular weights of 75, 80 and 60 kDa could be specifically crosslinked to γ, β1, and μ1, respectively. The identity and function of these proteins is still not known, however, nor whether there are equivalent proteins that interact with subunits of the AP-2 complex.

3.6. Regulation of recruitment

Information on the regulation of adaptor recruitment has come mainly from in vitro studies making use of either permeabilised cells or membrane fractions [63,96,121–124]. These studies have shown that the binding of adaptors to their target membrane is not a simple event. The addition of GTPγS to permeabilised cells stimulates the recruitment of AP-1 onto TGN membranes, while addition of the fungal metabolite, brefeldin A (BFA), prevents AP-1 recruitment in vitro and causes AP-1 to redistribute to the cytoplasm when added to living cells [122,125]. There is evidence that both GTPγS and BFA are acting via the ARF family of small GTP-binding proteins. GTPγS causes ARF to become constitutively activated, while BFA inhibits the nucleotide exchange of ARF [126]. More direct evidence for the involvement of ARF in AP-1 recruitment came from studies in which cytosol was depleted of ARF by gel filtration and AP-1 recruitment was found to be reduced, while full recruitment could be restored by the addition of recombinant myristoylated ARF1, the most abundant of the ARF isoforms [120,123,127]. Thus, it appears that TGN membranes need to be 'primed' with ARF1 before AP-1 can bind, although since ARF1 also primes the membranes of the intermediate compartment and the Golgi stack prior to coatomer binding, it is unlikely that it contributes to the specificity of AP-1 binding.

The priming of membranes by ARF1 may be due, at least in part, to its ability to activate phospholipase D (PLD) [128,129]. The addition of exogenous PLD stimulates the recruitment of coatomer (a component of the COPI coat) onto membranes, while inhibitors of PLD block coatomer recruitment and also interfere with membrane traffic between the Golgi stack and the ER [130,131]. However, neither exogenous PLD nor PLD inhibitors have any effect on the recruitment of AP-1 adaptors onto the TGN, suggesting that ARF may stimulate coatomer recruitment and AP-1 recruitment in different ways. There are also important differences between the regulation of AP-1 recruitment and AP-2 recruitment. The association of AP-2 with the plasma membrane is not affected by BFA, either in vitro or in vivo, suggesting that ARF1 is not involved [122,125,132]. However, GTPγS causes mistargeting of AP-2 adaptors to an endosomal compartment [132], and also inhibits the in vitro formation of clathrin-coated vesicles at the plasma membrane [133]. Certain cationic amphiphilic drugs also cause AP-2 to bind to endosomes without affecting AP-1 recruitment [134]. More recently, it has been shown that the addition of either constitutively active ARF1 or exogenous PLD also causes AP-2 adaptors to bind to the endosomal compartment. Neomycin, which inhibits PLD by binding to its cofactor PIP_2, inhibits the binding of AP-2 not only to the endosomal compartment but also to the plasma membrane, suggesting that both events require PLD. These findings indicate that the putative AP-2 docking site is somehow activated by PLD, but that normally this activation only occurs at the plasma membrane [124]. It is possible that the most divergent of the ARF isoforms, ARF6, may be involved in the normal recruitment of AP-2 since it has been localised to the plasma membrane [135] and is BFA-insensitive [136]. However, it is clear from these experiments that AP-2 recruitment is regulated differently from AP-1 recruitment.

There is also some evidence to suggest that phosphorylation of adaptor subunits may help to regulate not only the recruitment of adaptors onto mem-

branes, but also their ability to coassemble with clathrin [137], although AP-1 recruitment onto membranes has been shown to be ATP-independent, at least in vitro [34], suggesting that de novo phosphorylation is not required. In addition to the small GTP-binding proteins, heterotrimeric G proteins may also have a role in adaptor recruitment. The pre-incubation of cells with aluminium fluoride, which activates heterotrimeric G proteins, has been shown to prevent the BFA-induced redistribution of AP-1 from the TGN to the cytoplasm [122]. Incubation with another activator of heterotrimeric G proteins, mastoparan, inhibits the AP-2-stimulated sequestration of ligand in assays for clathrin-coated vesicle formation at the plasma membrane [133]. Certainly, the cell must have some means of sensing the amount of membrane in different compartments and thus ensuring that incoming and outgoing traffic are balanced, and at present GTP-binding proteins are the best candidates for proteins that might regulate vesicle budding.

3.7. Other protein interactions

So far, it has not been possible to reconstitute the entire process of clathrin-coated vesicle formation in vitro by adding a defined set of proteins to target membranes. This is in contrast to COPI and COPII coated vesicle formation, where the addition of the appropriate small GTP-binding protein and coat components is enough to drive vesicle budding [138,139], and it suggests that there are additional factors required for clathrin-coated vesicle formation, some of which have now been identified.

The first such protein to be discovered was dynamin, which in *Drosophila* is encoded by the *shibire* gene. Ultrastructural studies on cells from flies with a temperature-sensitive mutation in *shibire* showed that at the non-permissive temperature endocytosis was blocked because clathrin-coated pits were unable to pinch off as coated vesicles [140]. When the *shibire* gene was cloned and sequenced, the protein product was found to be 69% identical to rat dynamin [141,142], a protein that had previously been described as a GTPase mediating microtubule bundling in vitro [143], although more recent studies indicate that dynamin does not interact with microtubules in vivo. At least three isoforms of dynamin have been identified in mammals: dynamin-1, which is neurone-specific; dynamin-2, which is expressed ubiquitously; and dynamin-3, which is testis-specific [144,145]. Support for a role for dynamin in endocytosis in mammals came from studies on the expression of a dynamin mutant deficient in GTP binding and hydrolysis [146,147]. Overexpression of this mutant was found to block receptor-mediated endocytosis at a stage following coat assembly and invagination. No other trafficking pathways were affected, indicating that dynamin only acts at the plasma membrane. A model for how dynamin might act to sever the coated vesicle from the plasma membrane has been suggested, based on electron micrographs of synaptosomes incubated with GTPγS. In these preparations, the coated pits were found to have unusually long necks, ringed with structures resembling collars or necklaces, and immunogold labelling revealed that these collars were composed of dynamin [148]. Purified dynamin has been shown to self-assemble into rings which can then stack to form similar-looking structures [149]. The emerging model is that dynamin rings form a collar around deeply invaginated coated pits, which then closes to form free clathrin-coated vesicles.

There is evidence that a homologue of dynamin may play a similar role in the formation of clathrin-coated vesicles at the TGN, since an antibody raised against dynamin has been shown to cross-react with a 100 kDa band on Golgi membranes [150]. In addition, in yeast a dynamin homologue, Vps1p, is required for the export of vacuolar proteins from a late Golgi compartment [151]. Thus, it is possible that a dynamin-like protein is required for all clathrin-mediated budding events.

By gel overlay and affinity chromatography, a protein named amphiphysin has been shown to interact specifically with dynamin [112]. This protein was first identified as a novel SH3 domain-containing neuronal protein enriched in synaptic vesicles [152], and subsequently as the autoantigen of Stiff-Man syndrome, often associated with breast cancer [153]. Amphiphysin binds not only to dynamin, but also to the AP-2 adaptor complex via the α-adaptin ear domain, enabling it to be recruited to plasma membrane clathrin-coated pits. Like dynamin, amphiphysin was originally identified in neurones, but is now known to be expressed ubiquitously, with at

least two different genes and several different splicing variants. Amphiphysin binds not only to dynamin and α-adaptin, but also to synaptojanin [154], a phosphatase that cleaves PIP_2 and other inositol phospholipids which is also localised to clathrin-coated pits at the plasma membrane, although its function there is not known.

Another protein that binds to the α-adaptin ear is Eps15 [111,155], which was originally described as a substrate for the EGF receptor tyrosine kinase [156]. By immunogold electron microscopy, Eps15 has been localised to the edge of the forming coated pit, suggesting that like dynamin, it may have a role in vesicle budding [157]. Eps15 contains an evolutionarily conserved motif of ~70 amino acids, called the Eps15 homology (EH) domain [158]. There is evidence that the EH domain binds to proteins containing the sequence NPF [159], and although the precise function of the EH domain is not yet known, it has been found in a number of other proteins which are implicated in endocytosis, including the yeast proteins End3p [160] and Pan1p [161]. Another EH domain-containing protein, γ-synergin, binds to the C-terminal ear domain of the γ-adaptin subunit of the AP-1 complex [113]. However, the EH domain is not part of the adaptor binding site of either Eps15 or γ-synergin.

Two neuronal-specific proteins, AP-180 and auxilin, have been purified as minor components of brain clathrin-coated vesicles [162,163], and recently a ubiquitously expressed homologue of AP-180, CALM, has been identified [164]. CALM contains two copies of the sequence NPF, making it a candidate EH domain-binding protein. Both AP-180 and auxilin have been shown to promote clathrin assembly in vitro; indeed, AP-180 is four times more active than AP-1 or AP-2 [165], and presumably both AP-180 and CALM help to regulate clathrin assembly in vivo. However, in the case of auxilin, there is now evidence that its function in vivo might be to promote clathrin disassembly rather than clathrin assembly, since it has been shown to be a cofactor for the clathrin uncoating ATPase, Hsc70 [166].

The clathrin uncoating ATPase was first described as a factor that promotes ATP-dependent clathrin-coated vesicle uncoating in vitro [95]. It was subsequently purified and shown to be identical to Hsc70, a member of the Hsp70 family of chaperone proteins [167]. Hsc70 does not solubilize adaptors from clathrin-coated vesicles so there must be other factors involved in uncoating, but immunoprecipitation experiments carried out on cytosolic clathrin showed that Hsc70 co-precipitates, suggesting that it associates with non-assembled clathrin and possibly prevents inappropriate assembly. Another protein was also found to co-immunoprecipitate with cytosolic clathrin and was identified as valosin-containing protein (VCP) [168], although the significance of this interaction is not yet clear.

A recent study on the endocytosis of the β2-adrenergic receptor has raised the possibility that the association of clathrin with membranes may not always be mediated by AP complexes. Previous studies had shown that after the β2-adrenergic receptor is activated by an agonist, it binds to a protein called β-arrestin, and this is followed by its internalisation in clathrin-coated vesicles. In an in vitro system, β-arrestin was found to bind to clathrin cages, suggesting that it might serve to link the β2-adrenergic receptor to clathrin without the need of conventional adaptors [169]. Although there is as yet no evidence that this occurs in vivo, it is worth noting that in yeast, knocking out genes encoding AP subunits has a much less severe phenotype than knocking out clathrin heavy or light chain genes, suggesting that clathrin is at least partially functional even in the absence of APs.

4. Clathrin and adaptors in organisms

The clathrin heavy chain gene has been knocked out in two unicellular organisms, yeast [39,170] and *Dictyostelium* [171]. Surprisingly, both organisms are viable although growth is impaired. The phenotype has been more extensively studied in yeast, where two proteins normally resident in the TGN, Kex2p and DPAP A, have been found to be mislocalised to the plasma membrane. There is also a partial block in the receptor-mediated endocytosis of α-factor, although sorting of the vacuolar enzyme, carboxypeptidase Y (CPY), is unimpaired. However, in a temperature-sensitive clathrin heavy chain mutant, it was found that after the temperature shift CPY was initially missorted to the plasma membrane, but after several hours, normal sorting to the vacuole

was resumed. Thus, in yeast, it appears that sorting to the vacuole is normally clathrin-dependent, but that there is another mechanism that can be switched on if the clathrin pathway is blocked [172,173]. Clathrin light chain knockouts in yeast have a similar phenotype to clathrin heavy chain knockouts, although surprisingly, knocking out any of the four putative AP-1 subunit genes, either alone or in combination, does not cause any obvious sorting defects. However, there are synthetic effects between the clathrin and AP-1 genes in that the phenotype of the temperature-sensitive clathrin heavy chain mutation is exacerbated when one of the AP-1 genes is disrupted as well.

The clathrin heavy chain gene has also been knocked out in *Drosophila melanogaster*, and here it has been found to be essential [174]. *Drosophila* α-adaptin has also been knocked out, by P element insertion, and the flies were found to die early in embryogenesis, while an insertion in the non-coding region led to paralysis due to an inhibition of endocytosis and synaptic vesicle recycling at the neuromuscular junction [80]. Intriguingly, there is another *Drosophila* gene, *stoned*, with some homology to the adaptor μ subunit family, which when mutated also causes paralysis [175], although its homology is so distant that it is unlikely to be a subunit of a conventional AP complex. In the nematode worm, *Caenorhabditis elegans*, a naturally occurring mutation in μ1, *unc 101*, has been identified. The phenotype includes uncoordinated movements and abnormal development of the vulva [75]. In other organisms, clathrin and adaptors have been implicated in processes ranging from learning in the sea slug *Aplysia* [176] (based on the finding that clathrin light chain is one of the proteins whose synthesis is increased upon long-term potentiation) to maintenance of the architecture of the pollen tube tip in plants (based on immunofluorescence localisation) [177].

5. Adaptor-related complexes

There are many other membrane traffic pathways in addition to those mediated by the three well characterised types of coats, COPI, COPII, and clathrin plus adaptors, and this has led to the suggestion that more coats must exist to function in these other pathways [178]. In addition, sorting signals similar to those that facilitate endocytosis can function in other pathways as well, suggesting that there may be additional adaptor complexes or adaptor-like complexes that recognize these signals on different membranes [33,179].

The first novel adaptor-related proteins to be described were two medium chain homologues, originally called p47A and p47B and now renamed μ3A and μ3B [180]. μ3A and μ3B are 84% identical to each other and each is ∼26% identical to μ1 and μ2, which are themselves 38% identical. μ3A is expressed ubiquitously, while μ3B is neuronal-specific. The second such protein to be identified was a β homologue, originally called β-NAP (for neuronal adaptin-like protein) and now renamed β3B [181]. Again, β3B is less like β1 and β2 than β1 and β2 are like each other. Both β3B and the two μ3 isoforms were originally cloned using antisera to screen expression libraries. Immunoprecipitation experiments using antibodies against recombinant β3B and μ3 showed that β3 and μ3 are components of the same complex [34]), now known as the AP-3 complex. The other two subunits of the complex, δ and σ3, as well as a ubiquitously expressed β3 isoform, β3A, were all found by searching the EST database for proteins with the expected degree of homology to known AP subunits, and then using antibodies to show that all four subunits co-immunoprecipitate [35,36]. A functionally equivalent complex has also been shown to exist in yeast, encoded by the genes *APL5*, *APL6*, *APM3*, and *APS3* [84].

Immunofluorescence has revealed that the AP-3 complex is associated with the Golgi region of the cell and with more peripheral structures. The peripheral structures show limited colocalisation with endosomal markers and may correspond to a post-TGN biosynthetic compartment that can be at least partially accessed by endocytosed proteins [34–36]. Immunogold labelling using an antiserum against β3B indicates that some of the AP-3 is associated with the same membranes as AP-1, presumably the TGN compartment, although the two complexes are not associated with the same vesicular profiles [34]. Strikingly, AP-3 does not colocalize with clathrin at either the light or electron microscope level, and it is not enriched in purified clathrin coated vesicles [34–36].

The function of the AP-3 complex is just beginning

to be elucidated. Immunolocalisation indicates that it acts at a post-Golgi step, while studies making use of the yeast two-hybrid system have shown that μ3, like μ1 and μ2, binds to YXXØ sorting signals [35]. These types of sorting signals can be used in several different post-Golgi pathways, and may help to direct proteins to any one of several destinations, including the basolateral plasma membrane, endosomes, lysosomes, or back to the TGN [179]. Evidence that the AP-3 complex facilitates transport to a lysosome-like compartment comes mainly from genetic studies. In *Drosophila*, the δ subunit of the complex is encoded by *garnet*, an eye colour gene first described in 1915 [36,182,183]. Pigment granules in *garnet* mutants contain reduced amounts of both red and brown pigments, indicating that the *garnet* gene plays some sort of role in pigment granule formation. Since pigment granules have been shown to be similar to lysosomes in their origin, and since the complex is expressed ubiquitously, not just in pigment cells, this suggests a role in lysosomal biogenesis. Further evidence for such a role comes from studies on yeast. Deleting any of the yeast AP-3 genes specifically impairs the vacuolar delivery of alkaline phosphatase, a membrane protein, but not of CPY [84,184]. Thus, it seems likely that in all eukaryotes, AP-3 is used for the transport of a subset of proteins to lysosomes or lysosome-like organelles. Indeed, very recently mouse mutants have been found in two of the AP-3 subunits, and the phenotype is consistent with a role in sorting to lysosomes and related organelles, including melanosomes and platelet dense bodies [185].

Recent studies also suggest that in addition to AP-1, AP-2, and AP-3, there is at least one other AP complex in vertebrates. A novel member of the μ family has been found both in chickens and in mammals, and sequence comparisons indicate that the protein is a component of a novel complex rather than another isoform of one of the known μ subunits [186]. Searching the EST database shows that there are also novel members of the γ/α/δ, β, and σ families, although it is not yet known whether these are all components of the same complex. Two challenges for the future will be to correlate the new APs with particular pathways, and to find the (putative) protein or proteins taking the place of clathrin on vesicles coated with AP-3 and any other new AP complexes.

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