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Clathrin: Its Role in Receptor-Mediated Vesicular Transport and Specialized Functions in Neurons

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ABSTRACT: Clathrin constitutes the coat of vesicles involved in three receptor-mediated intracellular transport pathways; the export of aggregated material from the *trans*-Golgi network for regulated secretion, the transfer of lysosomal hydrolases from the *trans*-Golgi network to lysosomes and receptor-mediated endocytosis at the plasma membrane. The clathrin subunits and the other major coat constituents, the adaptor polypeptides, interact in specific ways to build the characteristic polygonal clathrin lattice and to attach the coat to integral membrane receptors. Both clathrin coat assembly and disassembly on the cytoplasmic side of the membrane are multistep processes that are regulated by the coat constituents themselves and by cytosolic proteins and factors. Neurons represent a cell type with distinct morphology and special demands on exocytic and endocytic pathways that requires neuron-specific constituents and modifications of clathrin-coated vesicles.

KEY WORDS: clathrin-coated vesicles, intracellular transport, mammals, receptor-mediated endocytosis.

I. INTRODUCTION

Transport of proteins, hormones, and nutrients between intracellular membrane-bound organelles and the plasma membrane allows eukaryotic cells to interact with their environment. Two major transport pathways can be distinguished: the exocytic, or secretory, pathway and the endocytic pathway (Figure 1) (Anderson, 1992; Rothman and Orci, 1992). The exocytic pathway serves to export material from the cell interior to the cell surface or to the extracellular environment, whereas the endocytic pathway imports material into the cell. Each pathway involves a distinct subset of intracellular membrane-bound organelles. The endoplasmic reticulum (ER) and the Golgi apparatus with its subcompartments are organelles associated with exocytosis. Endosomes and lysosomes are the compartments important for endocytosis. Connections between both pathways exist. One is the plasma membrane, which provides the endpoint of the exocytic pathway and the startpoint for the endocytic pathway. Another is the transport of newly synthesized lysosomal hydrolases from the exocytic pathway via the *trans*-Golgi network (TGN) to the lysosomes of the endocytic pathway.

Vesicles that bud from one compartment and fuse with the next create a vectorial transport along the pathways. Two modes of vesicular transport can be distinguished: constitutive and regulated. In the constitutive secretory pathway, material is nonselectively included into the transport vesicles and there it is present in the same concentration as in the organelle itself. This pathway is also referred to as the default or the bulk flow pathway (Rothman and Orci, 1992). In contrast, transport vesicles of the regulated secretory pathway divert selected proteins from the bulk flow pathway that require sorting. The vesicle contents are present in higher concentration than in their compartment of origin, resulting in efficient transport (Huttner and Tooze, 1989; Miller and Moore, 1990; Tooze, 1991). The constitutive and the regulated secretory pathways diverge at the TGN (Tooze and Huttner, 1990a). Examples of

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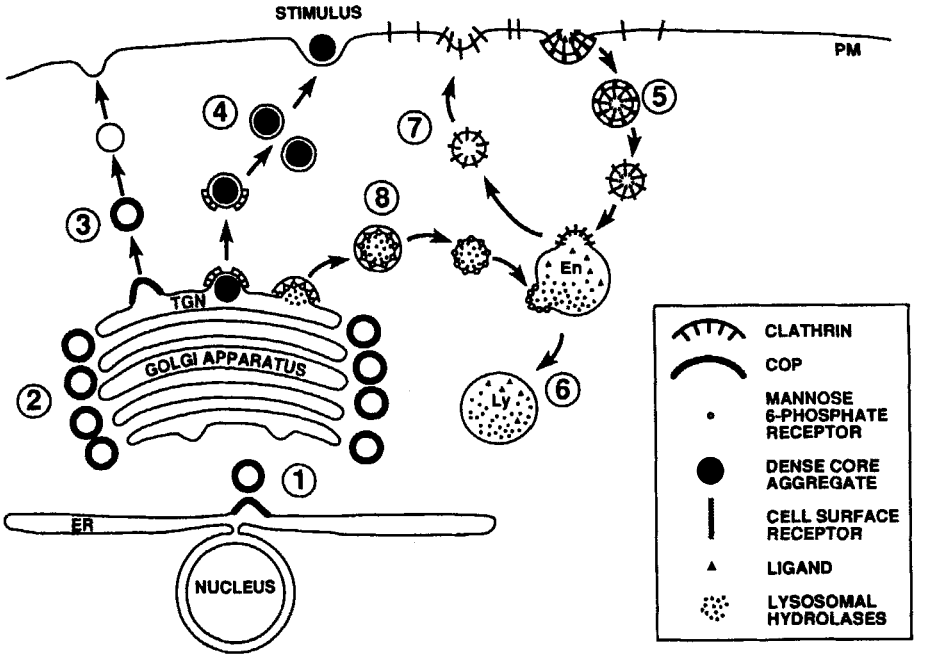


FIGURE 1. Intracellular vesicular transport pathways. Secretory pathway: (1) Newly synthesized proteins entering the secretory pathway by translocation into the ER are nonselectively transported via COP-coated vesicles from the ER to the *cis* side of the Golgi apparatus and (2) within the Golgi stack. (3) COP-coated vesicles form at the TGN and mediate constitutive secretion. In the regulated secretory pathway, partially clathrin-coated vesicles incorporate dense core aggregates formed in the TGN. Interaction of the aggregate with membrane receptors has not been established. Instead, clathrin might be involved in subsequent sorting and removal of materials from the secretory vesicle (Burgess and Kelly, 1987). (4) Secretory vesicles are stored in the cytoplasm prior to their stimulus-dependent fusion with the plasma membrane. Receptor-mediated endocytosis: (5) Receptor-ligand complexes cluster in clathrin-coated pits at the plasma membrane and are internalized. Endocytic vesicles fuse with endosomes in which receptor ligand complexes dissociate. (6) Ligands can be transported to lysosomes and be degraded, whereas (7) the receptors can recycle to the cell surface. Transport of lysosomal hydrolases: (8) Lysosomal hydrolases bind to mannose-6-phosphate receptors, concentrate into clathrin-coated pits at the TGN, and are transported to lysosomes. All COP- and clathrin-coated vesicles are uncoated prior to fusion with the target membrane. En, endosome; ER, endoplasmic reticulum; Ly, lysosome; PM, plasma membrane; TGN, *trans*-Golgi network.

molecules secreted in a regulated fashion are insulin in the β-cells of the pancreas and adrenocorticotropic hormone in pituitary cells (AtT20) (Orci et al., 1987; Tooze and Tooze, 1986; Tooze, 1991). A second sorting pathway that occurs in the TGN targets newly synthesized lysosomal hydrolases that bear a mannose 6-phosphate tag. These hydrolases interact with mannose 6-phosphate receptors in the TGN, causing their selective transport to lysosomes (Brown and Farquhar, 1984; Griffiths et al., 1988; Lemansky et al., 1987). Receptor-mediated endocytosis is a selective and regulated transport process that takes place at the plasma membrane. By specific interaction of ligands with receptors at the cell surface this process leads to internalization of many proteins (e.g., low-density lipoprotein), nutrients (e.g., iron bound to apotransferrin), hormones (e.g., insulin), and

certain viruses. Internalized endocytic vesicles fuse with endosomes. Proton pumps in endocytic membranes lead to acidification of the vesicle lumen and induce conformational changes in receptor ligand complexes, often leading to ligand dissociation. The endosome is capable of sorting ligands and receptors. Although receptors may be transported back to the cell surface, the ligands usually end up in lysosomes where they are degraded (Goldstein et al., 1985).

Most transport vesicles are transiently surrounded by a protein coat. Clathrin and adaptor complexes constitute the coat of vesicles in the sorting pathways and are involved in selecting and concentrating the vesicle contents (Brodsky, 1988). Coatomer proteins (COPs) are distinct from clathrin and form the coat of nonselective transport vesicles in the constitutive secretory pathway (Rothman and Orci, 1992). However, there are structural similarities between COPs and polypeptides of the adaptor complexes.

All clathrin-coated vesicles (CCV) draw from a common pool of cytoplasmic clathrin triskelions, three-legged structures composed of three clathrin heavy chains (HC) and three clathrin light chains (LC). Triskelions are the assembly units of the coat (Ungewickell and Branton, 1981). Additionally, two cytoplasmic pools of functionally distinct adaptor complexes exist, AP-1 and AP-2. AP-1 complexes are structural components of CCV forming at the TGN, whereas AP-2 complexes are exclusively associated with CCV originating at the plasma membrane (Ahle et al., 1988; Pearse and Robinson, 1990). Formation of a clathrin coat on the cytosolic side of the membrane occurs at a limited number of sites, which are called coated pits. At coated pits the membrane is slightly depressed. Coated-pit formation involves binding of adaptor complexes to the membrane to build the inner layer of the coat. Clathrin triskelions then polymerize onto the adaptor complexes forming a polygonal lattice. Receptors cluster in coated pits by specific interaction with adaptor complexes (Pearse and Robinson, 1990). Finally, the membrane invaginates and fully coated vesicles pinch off the membrane. In the cytoplasm, triskelions are subsequently released from the CCV. This reaction is thought to be catalyzed by the cytosolic heat shock cognate protein Hsc70 and therefore ATP dependent (Rothman and Schmid, 1986). Triskelions are thus recycled to undergo additional cycles of assembly and disassembly. The uncoated vesicles can then fuse with their specific target compartment.

All eukaryotic cells examined express clathrin; including organisms as diverse as the yeast *Saccharomyces cerevisiae* (Lemmon et al., 1991; Silveira et al., 1990), the slime mold *Dictyostelium discoideum* (O'Halloran and Anderson, 1992a), the protozoa *Trypanosoma* (Shapiro and Webster, 1989; Webster, 1989), plants (Huang et al., 1990; Lin et al., 1991; McIntosh et al., 1990), insects (Roth and Porter, 1964), and mammals (Jackson et al., 1987; Kirchhausen et al., 1987a, b). In all these organisms, the principal organization of the secretory and endocytic pathways appears to be the same. In *S. cerevisiae,* however, receptor-mediated endocytosis is limited and may be used for uptake of α mating pheromone. In addition, clathrin is required for Golgi retention of a protease, Kex2p, involved in the processing of the α factor pheromone. Although this may be a function of endocytosis, it may also represent a clathrin function in yeast with no obvious counterpart in mammals (Payne, 1990). Trypanosomes live in the bloodstream of certain mammals. As parasites, their interactions with the environment are extensive and they exhibit high levels of endocytic activity at their flagellar pocket (Webster, 1989).

Mostly, clathrin has been studied in mammals and yeast, although it was first discovered in insect cells (Roth and Porter, 1964). While studies of mammalian cells have focused on the identification of intracellular organelles and pathways by morphological and biochemical characterizations (Brodsky, 1988), those of yeast and *Dictyostelium* have emphasized genetic approaches to eliminate clathrin function (O'Halloran and Anderson, 1992b; Payne, 1990; Seeger and Payne, 1992). Although the organisms grow slowly, the viability of most clathrin-deficient strains of these simple eukaryotes suggests that clathrin facilitates formation of intracellular transport vesicles but is not absolutely required for vesicle generation (Payne, 1990). *In vitro* reconstitution systems for CCV budding and uncoating have also contributed to understanding the regu-

lation of the clathrin assembly/disassembly cycle (DeLuca-Flaherty et al., 1990; Schmid, 1992).

II. CLATHRIN STRUCTURE

Three clathrin HCs and three clathrin LCs associate to form a clathrin triskelion, the assembly unit of the clathrin coat. They polymerize on the cytoplasmic site of membranes onto adaptor complexes associated with cytoplasmic tails of integral membrane receptors.

A. Clathrin Heavy Chain

Clathrin HC is ubiquitously expressed in all eukaryotic cells and full-length cDNAs have been cloned and sequenced from rat (Kirchhausen et al., 1987a), *Dictyostelium discoideum* (O'Halloran and Anderson, 1992a), and *Saccharomyces cerevisiae* (Lemmon et al., 1991). Partial cDNA sequences are also available from cattle and human (Dodge et al., 1991; Kirchhausen et al., 1987a). The rat clathrin HC contains 1675 amino acids and has a predicted molecular weight of 192 kDa. This value is slightly higher than the apparent molecular weight of 180 kDa observed in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Sequence comparisons show that clathrin HCs are highly conserved among mammalian species (99% identity for the sequences available from partial clones. Also, the sequence similarities to the yeast HC (50%) and *Dictyostelium* HC (57%) are remarkably high. Comparison along the length of the HC polypeptide reveals less homology in the N- (residues 1–479) and C-terminal regions (residues 1630–1675) than in the central region (residues 480–1629).

B. Clathrin Light Chains

Clathrin LC cDNA and protein sequences have been analyzed from three mammalian species (rat, cattle, and human) and from the yeast *S. cerevisiae* (Jackson and Parham, 1988; Jackson et al., 1987; Kirchhausen et al., 1987b; Silveira et al., 1990). Whereas *S. cerevisiae* only expresses one clathrin LC (Payne and Schekman, 1985; Silveira et al., 1990), mammals express two types, LCa and LCb, which are encoded by distinct single copy genes. In humans, the LCa and LCb genes are located on different chromosomes (Jackson and Parham, 1988).

Mammalian LCa genes undergo differential mRNA splicing. In all tissues except neurons, proteins of 213–218 amino acids in length are expressed (Wong et al., 1990). In neurons, alternate splicing yields two additional forms of LCa, a minor neuron-specific form with an insertion of 18 amino acids and a major neuron-specific form with an additional insertion of 12 amino acids at the same site (Table 1). This results in protein sizes of 231–236 amino acids (depending on the species of mammal) for the minor neuronal form and 243–248 amino acids for the major neuronal form. For LCb, differential splicing yields a nonneuronal form of 210–211 amino acids (again depending on species) and a neuron-specific form with an 18 amino acid insertion at the site homologous to the insertion site in LCa (Jackson et al., 1987; Kirchhausen et al., 1987b). The yeast clathrin LC polypeptide is 233 amino acids long (Silveira et al., 1990).

Although the cDNA and protein sequences of LCa, LCb, and yeast clathrin LC can be well aligned along most of their length, certain exceptions are notable. First, close to the 5′ end of the cDNA (nt 93–131), LCa contains a duplication of a sequence corresponding to nt 228–266 of the LCa cDNA and nt 302–340 of the LCb cDNA.* The latter two regions represent homologous parts of the coding region. The 5′ end of the LCb gene does not contain this duplication. Interestingly, the duplication in the 5′ end of the LCa gene is more homologous to the LCb sequence (29 out of 38 nt are identical) than to the LCa sequence (21 of 38 nt are identical) (Jackson and Parham, 1988). Second, a region in the 3′ end of the yeast LC gene (corresponding to amino acids 218–238) is homologous to the neuron-specific sequence in mammalian LCb, amino acids 165–186 (Silveira

* Nucleotides are counted from the beginning of the human brain type LCa and LCb cDNA clones, respectively (Jackson and Parham, 1988).

TABLE 1
Structural Components of Mammalian Clathrin-Coated Vesicles

Protein complex	Polypeptide	Polymorphism	Molecular weight (kDa)[a]	Cell type(s)
Triskelion	Clathrin heavy chain		192	Ubiquitous
	Clathrin light chain LCa	No insert	27.5	Ubiquitous
		30 Residue insert	31.5	Neurons
		18 Residue insert	29	Neurons
	Clathrin light chain LCb	No insert	27	Ubiquitous
		18 Residue insert	29	Neurons
AP-1	β′-Adaptin		115#	Ubiquitous
	γ-Adaptin		91	Ubiquitous
	47-kDa Polypeptide		48	Ubiquitous
	20-kDa Polypeptide		19	Ubiquitous
AP-2	β-Adaptin	b, No insert	104.5	Ubiquitous
		b, 14 Residue insert	106	Brain
		b*	115#	?
	α-Adaptin	α_A, 41 residue insert	107	Brain
		α_A, 19 Residue insert	105	Ubiquitous
		α_C	104	Ubiquitous
	50-kDa Polypeptide		50	Ubiquitous
	17-kDa Polypeptide		17	Ubiquitous
—	AP-3		91.4	Neurons
—	Auxilin		86#	Brain
—	p140		140#	Brain
Tubulin	α-Tubulin		50	Ubiquitous
	β-Tubulin		50	Ubiquitous

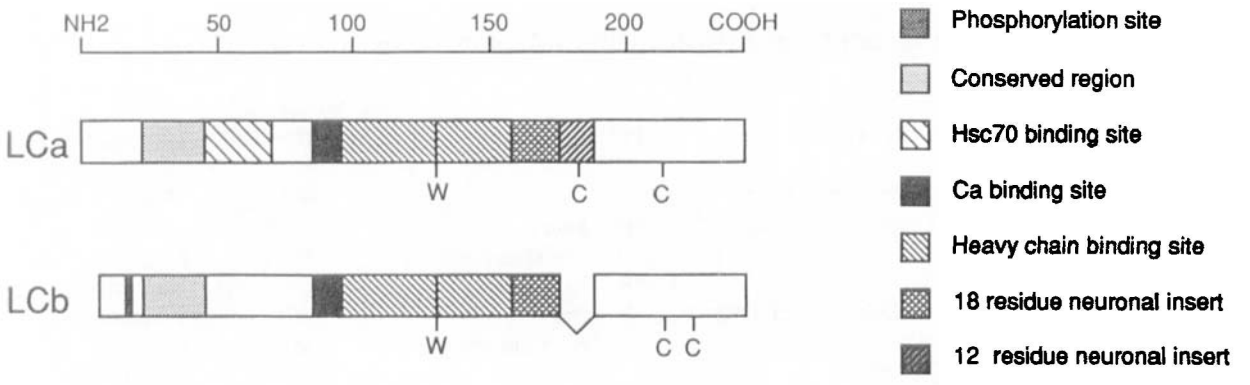
[a] Molecular weights are calculated from cDNA sequences, except for those indicated by #, which are molecular weights observed in SDS-PAGE.

et al., 1990). These similar sequences are located at non-homologous positions in the two genes.

Yeast and mammalian clathrin LCs exhibit 18% similarity in amino acid sequence (Silveira et al., 1990) and thus are more divergent than their respective HCs. This difference suggests there are greater constraints on the HC sequences to preserve their interactions in assembled clathrin than for the LCs. Conversely, the mammalian LCs may have evolved to fulfill different CCV functions than those served by the yeast LC. This concept can be extended to explain the divergence of the mammalian LCa and LCb genes. These genes seem to be an assembly of sequence motifs with each motif encoding a functional domain of the protein. Some of these domains are common to LCa and LCb, whereas others are unique to one or other polypeptide. Overall, LCa and LCb are 60% identical in protein sequence. The arrays of functional sites are thought to regulate aspects of clathrin function such as assembly and disassembly (Brodsky et al., 1991). Thus, the presence of two different LCs with different sets of functional domains in mammals compared with the one in yeast may offer diversity in regulatory capacity (Brodsky et al., 1991; Jackson and Parham, 1988). The sequence similarity of LCa (>96%) and LCb (>95%) in human, rat, and cattle indicates that functional diversification of the two LCs is a conserved feature in mammals (Jackson and Parham, 1988).

A description of the functional domains of mammalian LCa and LCb (Figure 2A), including the yeast LC where domains are shared, follows. The analysis proceeds from the amino- to the carboxy-terminus.

Phosphorylation is an LCb specific regulatory element that potentially regulates the formation of secretory granules, or rapid recycling of membrane after stimulated secretion, in cells possessing a regulatory secretory pathway. Such cells express five times as much LCb than LCa (Acton



A

FIGURE 2. **(A)** Schematic diagram of mammalian LCa and LCb domain structure. Identified structural and functional domains are listed on the right-hand side progressing from the N-terminus toward the C-terminus. The positions of the tryptophan skip residue (W) dividing the HC-binding site and the cysteine residues (C) capable of disulphide bond formation are indicated. **(B)** Triskelion structure. Three clathrin HCs (the legs) radiate symmetrically from the triskelion vertex. A bend in each leg (the knee) subdivides each leg into a proximal and a distal segment. The terminal domains are joined to the legs via a hinge. The LCs are located on the proximal segment close to the vertex and are shown folded into a U-shape with the tryptophan residue (W) in the turn. The two helices in the LCs involved in HC binding are depicted as hatched boxes. The N- and C-termini of the HCs and the LCs are indicated. **(C)** Structure of clathrin-coated vesicles. (1) Polygonal clathrin lattice composed of hexagons and pentagons. The position of one triskelion is shown to illustrate how each leg spans two edges. (Reproduced from Brodsky et al., *TIBS*, 16, 208–213, 1991. With permission.) (2) Association of triskelions within the clathrin lattice. Each lattice edge contains two proximal and two distal leg segments. At the edges of the proximal leg segments of the triskelion located in the center of the diagram (shaded) are proximal leg segments that originate from triskelions (bold) located at the next vertex and that are antiparallel to those of the central triskelion. Two distal leg segments in antiparallel orientation are present in each edge, one of which belongs to a triskelion centered one vertex away (bold) from the center triskelion (shaded) and the other to a triskelion centered two vertices away (plain line drawing). The three terminal domains located at the vertex of the central triskelion (shaded) belong to triskelions (plain line drawing) centered at the next but one vertices. (3) Cross-section of a clathrin-coated vesicle. Receptor-ligand complexes are located in the vesicle membrane. Adaptor complexes form the inner layer of the coat. Their ears interact with the receptor tails and their core binds to the terminal domains of clathrin triskelions, which form the outer layer of the coat.

and Brodsky, 1990), whereas cells engaging solely in constitutive secretion express similar levels of LCa and LCb. LCb can be phosphorylated *in vivo* and *in vitro* (Bar-Zvi and Branton, 1986; Bar-Zvi et al., 1988; Schook and Puszkin, 1985). Two *in vitro* phosphorylation sites have been mapped to serine residues 11 and 13 close to the N-terminus of the LCb polypeptide (Hill et al., 1988). These sites and the surrounding LCb sequences are conserved between species (Jackson et al., 1987; Kirchhausen et al., 1987b) and correspond to a casein kinase II target sequence (Kuenzel et al., 1987). Casein kinase II activity is associated with clathrin-coated vesicles and might reside in an intrinsic coated-vesicle protein. No corresponding serine residues are present in LCa explaining the absence of phosphorylation in LCa.

Amino acid residues 22–25 GEED in LCa and AEED in LCb (numbering for this and the following regions refers to the bovine major neuronal LCa sequence [Jackson and Parham, 1988]) are responsible for the anomalous slow mobility of clathrin LCs in SDS-PAGE. This small cluster of acidic amino acid residues may affect SDS binding or cause a nonlinear LC conformation in the electrophoresis (Näthke et al., 1990; Scarmato and Kirchhausen, 1990).

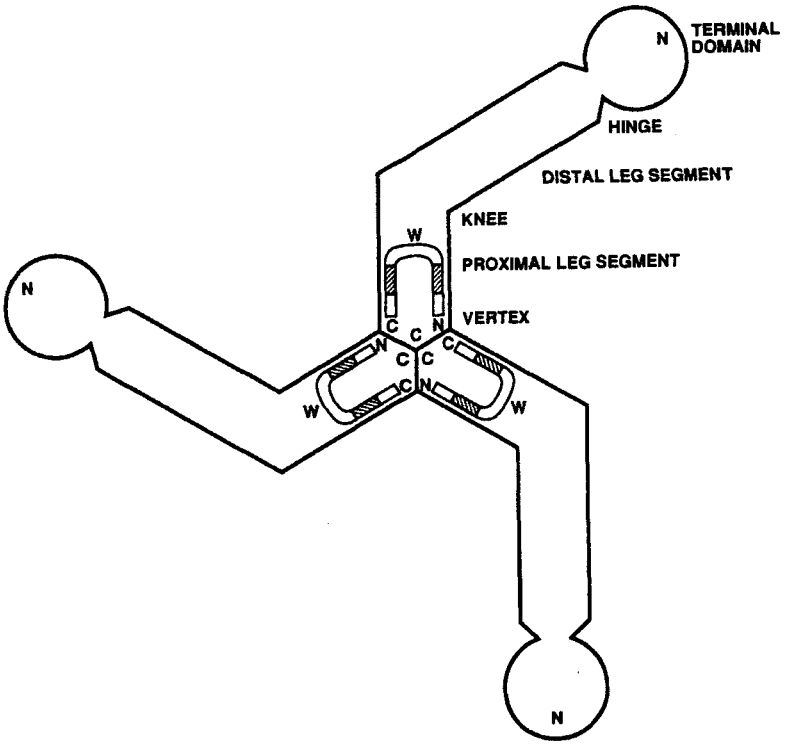


FIGURE 2B

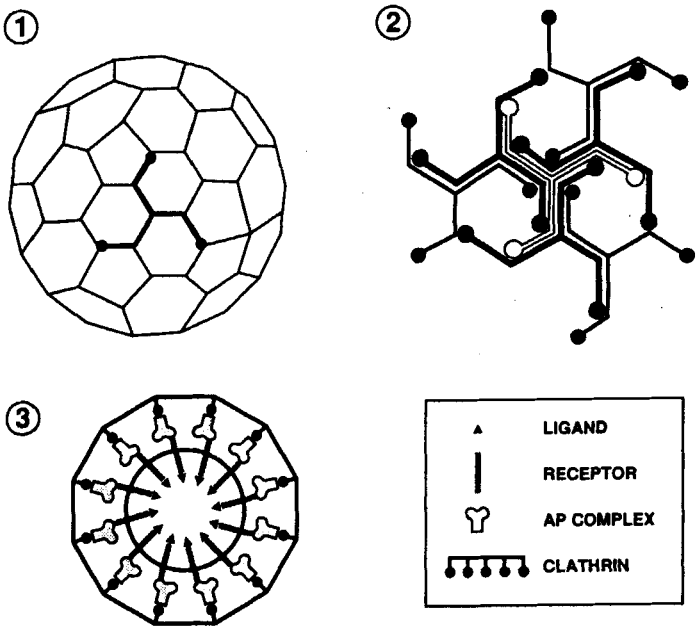


FIGURE 2C

Residues 23–44 are identical in all mammalian LCa and LCb polypeptides sequenced so far (Jackson and Parham, 1988; Jackson et al., 1987; Kirchhausen et al., 1987b). This sequence as well as the corresponding region in the yeast LC (Silveira et al., 1990) has a high probability for α-helical coiled coil formation (Näthke et al., 1992) but as yet has no assigned function.

Uncoating of clathrin-coated vesicles is mediated in an ATP-dependent fashion by the cytosolic heat shock cognate protein Hsc70. This reaction is dependent on the presence of clathrin LC (Rothman and Schmid, 1986; Schmid et al., 1984). Although both LCa and LCb bind to Hsc70 and promote ATP hydrolysis *in vitro*, LCa is more effective and may be more important for disassembly of coated vesicles (DeLuca-Flaherty et al., 1990). The site of interaction with Hsc70 maps to residues 47–71 of LCa, a stretch of amino acids rich in proline and glycine (DeLuca-Flaherty et al., 1990).

Adjacent to the Hsc70 binding site, residues 85–96 of both LCa and LCb comprise a calcium binding EF-hand motif (Näthke et al., 1990). Within the loop of this helix-turn-helix motif, amino acids D85, Y89, A91 (carbonyl from the backbone), and D96 chelate the Ca^{2+}. The yeast clathrin LC has a similar sequence motif at the homologous position. In yeast, the Ca^{2+} is chelated by D85, N87, S89, T91 (carbonyl from the backbone), and N96 (Näthke et al., 1990). Both light chains, LCa and LCb, bind Ca^{2+} with similar affinity, the kDa is 25 μM. This affinity is about one tenth lower than the affinity of other Ca-binding proteins with high affinity EF-hand motifs such as calmodulin or parvalbumin (Mooibroek et al., 1987).

Amino acid residues 93–157 mediate binding of the clathrin LCs to the clathrin HC (Brodsky et al., 1987; Jackson et al., 1987; Kirchhausen et al., 1987b; Scarmato and Kirchhausen, 1990). This region is predicted to form α-helical coiled coils when interacting with another polypeptide (Jackson et al., 1987; Kirchhausen et al., 1987b). Such sequences have heptad repeats in which the first and fourth residues of each repeat are hydrophobic, resulting in an amphipathic α-helix. In mammalian and yeast clathrin LC, ten heptad repeats are present in the HC-binding region. Between repeats five and six, there is a discontinuity due to the presence of an additional amino acid, tryptophan 130, called the skip residue (Jackson and Parham, 1988; Kirchhausen et al., 1987b; Silveira et al., 1990).

A stretch of hydrophobic amino acid residues (158–188) is located C-terminal of the HC-binding domain. These sequences are 18–30 amino acids in length and are expressed only in neurons. Yeast clathrin LC does not possess a region that corresponds in location directly to the neuron-specific sequences in LCa or LCb. Amino acid residues 218–238 of the yeast LC located further toward the C-terminus of the polypeptide, however, are reminiscent of the LCb neuron-specific sequence (Silveira et al., 1990). Because yeast are unicellular eukaryotes it is unknown what, if any, the functional significance of this sequence is.

Yeast as well as mammalian clathrin LCs are able to bind to calmodulin (Linden et al., 1981), which is a mediator of Ca-regulated processes. The three-dimensional conformation and the C-terminus of LCa (residues 189–243) are important for interaction with calmodulin (Pley et al., 1991; and unpublished results). Two segments within this region have particular homology (39%) to the yeast LC and may contribute to calmodulin binding. Residues 188–208 are exposed at the surface of triskelions and clathrin-coated vesicles as shown by their accessibility to the monoclonal antibodies CVC.6 and CVC.7 (Brodsky et al., 1987) and could directly contact calmodulin. Clathrin-coated vesicles and triskelions purified from bovine brain also bind to calmodulin, presumably via the LCs, but the functional relevance of this interaction is unknown.

Cysteine residues in the C-terminus of LCa (C182 and C223) are capable of transient disulphide bond formation *in vitro* giving rise to heterogeneity in banding pattern on SDS-PAGE (Parham et al., 1989).

Common to mammalian LCs and the yeast LC is their heat stability. This characteristic allows for readily prepared LCs by boiling preparations of clathrin triskelions or CCV (Holmes et al., 1984; Lisanti et al., 1982; Silveira et al., 1990).

C. Clathrin Triskelions

Triskelions (Figure 2B) are the assembly units of clathrin coats (Ungewickell and Branton, 1981). In electron micrographs, triskelions appear as three-legged structures joined at the center or vertex. Each leg is about 45 nm in length and morphologically three regions are distinguishable. A bend, the knee, near the midpoint subdivides a leg into a 17-nm-long proximal segment (located in proximity to the vertex) and a 25-nm-long distal segment. A globular domain, termed the terminal domain, is joined to the distal segment at the far end from the vertex. Besides the globular terminal domain and a short narrow joint that attaches the terminal domain to the leg (Kirchhausen et al., 1986), the legs appear to have a uniform mass distribution (Kirchhausen and Harrison, 1981). Triskelions are nonplanar, they possess a convex top and a concave bottom side. On the convex side, which faces the cytoplasm in assembled coats, the triskelions appear puckered at the vertex (Kirchhausen et al., 1986). When viewed from the top, all legs are bent in a clockwise direction at the hinge between the proximal and the distal leg segments (Kirchhausen et al., 1986).

Molecular weight determination (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981) and cross-linking studies (Kirchhausen and Harrison, 1981) revealed that each triskelion leg consists of one clathrin HC complexed to one LC. Whereas the HCs interact with each other at the vertex, the LCs do not contact each other within the triskelion (Kirchhausen and Harrison, 1981). Correlation of the clathrin HC primary structure with characterized tryptic cleavage sites (Kirchhausen and Harrison, 1984; Kirchhausen et al., 1987a) allowed the HC to be oriented within the triskelion. The N-terminus is in the terminal domain (residues 1–479) and the C-terminus is at the vertex (Kirchhausen et al., 1987a).

Investigation to characterize the HC-LC interaction has been performed along several lines. Proteases like trypsin, chymotrypsin, and elastase digest and release the LCs of triskelions and coated vesicles indicating they are exposed on the triskelion surface. These proteases also cleave the clathrin HC. A predominant cleavage occurs at the joint of the distal leg segment and the 52-kDa terminal domain, resulting in release of the terminal domain (Kirchhausen and Harrison, 1984). 110–125-kDa proteolytic HC products, corresponding to the proximal leg segments and the knee region, stay trimeric and are capable of rebinding clathrin LCs (Ungewickell, 1983; Winkler and Stanley, 1983).

Independently, the exposed location of the clathrin LCs and their binding to the proximal leg segment were confirmed by antibody-binding studies. LC-specific IgG were found to decorate the proximal leg of triskelions when viewed in electron micrographs (Kirchhausen et al., 1983; Ungewickell, 1983). Additionally, two monoclonal antibodies, X19 and X35, directed against the clathrin HC affect LC binding to the HC. X35 binds better to the HC when the LCs have been removed and loss of the X35 epitope by proteolytic digestion coincidentally abolishes the ability of the truncated HC fragment to bind LC (Blank and Brodsky, 1987). The X35 and X19 epitopes map to residues 1438–1481 of the clathrin HC, a region localizing to the proximal leg segment within 10 nm of the vertex (Näthke et al., 1992). Residues 1460–1489 are predicted to possess a high potential for forming an α-helical coiled coil that may mediate LC binding (Näthke et al., 1992). A weak pattern of eleven heptad repeats at residues 1107–1184 of the HC has also been suggested to mediate LC binding (Kirchhausen et al., 1987a). Although this sequence might contribute to LC interaction, it is not sufficient because tryptic HC fragments containing residues 1107–1184, but lacking residues 1438–1481, are unable to associate with LCs (Näthke et al., 1992).

Although isolated LCs contain little defined secondary structure in solution (Ungewickell, 1983), they are predicted to adopt a U-shaped conformation on binding to the HC. The bend may be centered on the skip residue W130 that splits the predicted HC-binding domain of the LC into two coiled-coil regions of five heptad repeats. Together, the data indicate that the two coiled-coil regions in the LC sequences, HC region 1460–1489 and possibly region 1151–1178 form a three- or four-helix bundle (Näthke et al., 1992). One turn of two α-helices forming a coiled-

coil dimer corresponds to ~80 amino acids for each helix. In the model for HC-LC interaction of Näthke et al. (1992) the length of each LC helix in the bundle is at maximum 35 amino acids long. This would allow for about half a coiled-coil turn and may best be imagined as the LCs clamping onto the HC. Such a model may explain the ease with which LCs can rebind to HC triskelions devoid of LC by changing to the U-shaped conformation. If the LCs do not have to fully wrap around the HC one can also explain how LCs can rebind to LC-free cages in which triskelions are in tight and extensive contacts with each other (also see later). The U-shaped conformation of the LC molecule when bound to the HC further implies that the functional domains located N-terminal (phosphorylation, Hsc70 binding, and Ca-binding) are in close proximity to the domains located C-terminal (neuron-specific sequences and calmodulin-binding site) of the HC-binding domain (Figure 2B). This juxtaposition may result in interaction between these sites.

Measurements of the affinity of the HC-LC interaction have given different results: K_A of 5×10^7 *M* by Ungewickell (Ungewickell, 1983) and K_A of 10^{10} M by Winkler and Stanley (Winkler and Stanley, 1983). The off rate for the interaction appears to be slow (Scarmato and Kirchhausen, 1990; Winkler and Stanley, 1983). Both light chains, LCa and LCb, are predicted to bind to the HC in a similar manner and appear randomly distributed on triskelions (Kirchhausen et al., 1983). Although only 60% identical in sequence (Jackson and Parham, 1988), they possess similar structures, as determined by circular dichroism (Winkler and Stanley, 1983) and compete for the same binding site on the HC (Ungewickell, 1983). Additionally, the HC-LC interaction has been conserved across species as demonstrated by heterologous binding of LCs and HCs from different mammalian sources (Ungewickell, 1983). Furthermore, a series of heptad repeats divided by the W130 skip residue is conserved in all LCs (Jackson and Parham, 1988; Silveira et al., 1990). Similarly, the rat HC region 1460–1489 that is required for LC binding also has a high probability for coiled-coil formation in *Dictyostelium* and *Saccharomyces* (O'Halloran and Anderson, 1992a; Silveira et al., 1990). In contrast, the region of 11 heptad repeats (residues 1107–1184) in the rat HC is not predicted to form an α-helical coiled-coil in the yeast HC (Näthke et al., 1992). Thus, although the HC-LC interaction may involve region 1107–1184 in *Dictyostelium* and mammals to form a four-helix bundle, this region may not participate in LC binding in yeast that might therefore involve a three-helix bundle (Näthke et al., 1992).

Recent proteolysis experiments have identified HC fragments of known size and sequence that elute from a HPLC-sizing column at positions corresponding to a molecular weight consistent with their presence as a trimerized complex. This analysis implicated amino acid residues 1522–1572 of the rat HC as the region that mediates trimerization (Näthke et al., 1992). The positive localization of the trimerization domain to this region and not the C-terminus of the rat HC agrees with the finding in yeast showing that a deletion of the C-terminal 57 amino acids does not abrogate the ability of the yeast HC to trimerize (Lemmon et al., 1991). The HC-trimerization domain is predicted to adopt an α-helical conformation but the precise interactions between such helices in a triskelion are presently unknown (Näthke et al., 1992). The HCs of *Saccharomyces* and *Dictyostelium* contain a homologous region (Näthke et al., 1992), indicating a similar trimerization mechanism. In contrast, the region C-terminal of the proposed trimerization site in HCs from different species is variable in length and divergent in sequence. The three HC C-terminal sequences in a triskelion may form small globular domains on top of the vertex (Kirchhausen et al., 1986; Näthke et al., 1992). Although the variability in the C-terminal domains has been speculated to fulfill species-specific functions in yeast, *Dictyostelium*, and mammals, this hypothesis is as yet untested.

D. Adaptor Complexes

Four different classes of clathrin-associated proteins have been identified and are termed AP-1 (HA-I), AP-2 (HA-II), AP-3 (AP180, NP185), and auxilin (Ahle and Ungewickell, 1986, 1990; Keen, 1990; Keen and Black, 1986; Kohtz and Puszkin, 1988; Pearse and Robinson, 1984). The adaptor complex AP-1 that is

associated with CCV at the TGN and the plasma membrane-associated AP-2 complex are present in all cell types and were originally distinguished by their differential binding to hydroxylapatite (hence the original terminology HA-I and HA-II) (Pearse and Robinson, 1984). AP-3 and auxilin are expressed only in brain and are discussed in more detail in the section about clathrin in neurons (Ahle and Ungewickell, 1986, 1990; Keen and Black, 1986; Kohtz and Puszkin, 1988).

Chemical cross-linking studies and gel filtration chromatography demonstrated the molecular weight of the AP-1 and AP-2 complexes to be 250–340 kDa (Keen, 1987; Manfredi and Bazari, 1987; Pearse and Robinson, 1984; Virshup and Bennett, 1988). Each consists of four distinct polypeptides. For AP-2, these comprise two chains of a group of 100–115 kDa polypeptides, called α and β adaptin, as well as 50 kDa- and 17 kDa- (sometimes referred to as 16 kDa-) polypeptides (Ahle et al., 1988; Manfredi and Bazari, 1987; Virshup and Bennett, 1988; Zaremba and Keen, 1983). The AP-1 complex has a similar composition of related polypeptides, containing 100–115 kDa β′ and γ-adaptins and polypeptide species of 47 and 20 kDa (sometimes called 19 kDa) (Ahle and Ungewickell, 1988; Keen, 1987; Manfredi and Bazari, 1987).

The stoichiometry of the proteins in the respective AP-1 and AP-2 complexes has not been resolved unequivocally. Cross-linking has shown the α and β adaptins in the AP-2 complex directly contact each other (Pearse and Robinson, 1984; Virshup and Bennett, 1988). Also, α and β adaptin are present in equimolar amounts in the AP-2 complex. In conjunction with the molecular weight of the complex this observation suggests the AP-2 complex contains one copy each of α and β adaptin. However, the molecular weight of the native AP-2 complex and the molar ratio of the 100–115 kDa adaptins to the 50-kDa subunit assessed by various groups lead to structural proposals favoring the presence of either one (Matsui and Kirchhausen, 1990; Virshup and Bennett, 1988) or two (Keen, 1990; Manfredi and Bazari, 1987; Pearse and Robinson, 1984) copies each of the 50-kDa and 17-kDa polypeptides in a single AP-complex. Freeze-etch electron microscopy revealed the AP-2 complexes to be a massive bricklike structure with two smaller earlike appendages (Heuser and Keen, 1988; Virshup and Bennett, 1988). The ears can be released from the brick by proteases such as elastase (Heuser and Keen, 1988) and trypsin (Matsui and Kirchhausen, 1990). Biochemical analysis has shown that the ears comprise 30–35-kDa fragments of α and β adaptin, respectively. The 50-kDa and 17-kDa polypeptides remain in the core in association with the 70-kDa proteolytic fragments of α and β adaptin (Heuser and Keen, 1988; Matsui and Kirchhausen, 1990). Within the core, the 50-kDa and the 17-kDa polypeptides do not seem to contact each other but are in close proximity to the adaptins (Virshup and Bennett, 1988).

Among the α adaptins of the bovine AP-2 complex, various subtypes have been characterized biochemically and immunologically. One group identified two subtypes of α, termed αA and αC (Robinson, 1987), whereas another group resolved four α polypeptides a1, a2 (each 112 kDa), c1 and c2 (each 104 kDa) (Ahle et al., 1988). The exact relationship between the subtypes found by these two groups is unclear. Because of their relative molecular weights, αA might correspond to the a1/a2 α adaptins whereas αC resembles c1/c2. For the β adaptins of the AP-2 complex, two forms called b and b* are distinguishable (Ahle et al., 1988).

Amino acid sequences have been deduced from cDNA clones for the mammalian α-, β-, and γ-adaptins, as well as the 50-, 47-, 17-, and 20-kDa subunits (Kirchhausen et al., 1991, 1989; Nakayama et al., 1991; Ponnambalam et al., 1990; Robinson, 1989, 1990; Thurieau et al., 1988). The αA and αC adaptins are encoded by two different single copy genes that are 84% identical at the amino acid level (Robinson, 1989). It is unlikely that more genes encoding α-adaptins are present in the genome and therefore it is unknown how the a1, a2, c1, and c2 subtypes arise. Possibilities include alternative splicing events or posttranslational modifications, for example, phosphorylation. For β-adaptin, two clones have been isolated that are products of two different genes. One sequence corresponds to the b form of the AP-2 β-adaptin, whereas the identity of the second clone is unclear (Kirchhausen et al., 1989). The b form β-adaptin gene can undergo brain-specific splicing (Ponnambalam et al., 1990), which is discussed later (see section about clathrin in neu-

rons). Only for β and β′ adaptins tryptic digestion indicated that the polypeptides are related in sequence to each other. cDNA sequence analysis confirmed this and established that α and γ adaptins are homologous polypeptides within the adaptin family (Robinson, 1990) despite the absence of common tryptic fragments (Ahle et al., 1988). Additionally, the 50-kDa and 47-kDa polypeptides exhibit sequence similarities as do the 17-kDa and 20-kDa proteins. These observations indicate that the AP-1 and AP-2 complexes are structurally homologous complexes in which the homologous proteins occupy similar positions. Correlation of the primary structures deduced from cDNA clones of all the 100–115-kDa adaptins with amino acid sequences from the tryptic 70-kDa and 30-kDa adaptin fragments indicated that the polypeptide N-terminus is located in the AP brick, whereas the C-terminus corresponds to the ear. Within the adaptin sequences, the amino terminal domains of the AP brick are more closely related to each other than are the C-terminal domains that form the ears. Additionally, the α and γ adaptins differ in the size of their ear domains. These divergences suggest the ears may mediate AP-1 and AP-2-specific functions (discussed later in the section about Assembly). The site in the polypeptide sequence expected to contain the proteolytic cleavage site between the brick and the ear is rich in proline and glycine in all adaptins and possibly provides a flexible hinge between the two domains (Ponnambalam et al., 1990).

Although AP proteins have not been identified in yeast, genes homologous to the mammalian AP complex genes have been found. A homolog of the mammalian β adaptin gene possesses an open reading frame encoding an 80-kDa polypeptide. Its sequence is related to mammalian β adaptin along the entire length of the N-terminal putative core domain but lacks a counterpart of the proline/glycine-rich hinge region and completely diverges in the C-terminal domain (Kirchhausen, 1990). A predicted 54-kDa yeast homologue to the medium chain length components of the AP complexes has a closer relationship to the 47-kDa protein of the AP-1 complex (56% sequence identity) than to the 50-kDa component of AP-2 (39% sequence identity) (Nakayama et al., 1991). In contrast, the predicted yeast homolog of the small chain is more similar to the 17-kDa protein of AP-2 than to the 20-kDa polypeptide of AP-1 (Kirchhausen et al., 1991). If yeast possesses functional adaptor complexes *in vivo*, it remains to be seen whether an α- or γ-like adaptin protein exists, as found in mammals.

E. Clathrin-Coated Vesicles

In cells, CCV form on the cytoplasmic sides of the plasma membrane and the TGN (Anderson et al., 1977; Bleil and Bretscher, 1982; Brodsky, 1988; Goldstein et al., 1985). Their principal structural components are the vesicle lipid bilayer with its associated peripheral and integral membrane proteins, the adaptor complexes, and the clathrin triskelions. To elucidate the structure of CCV, related structures, in addition to purified clathrin-coated vesicles, have been studied *in vitro*. These include clathrin cages and clathrin coats. Clathrin cages are composed exclusively of triskelions that may or may not have clathrin LCs bound. Clathrin coats are structures consisting of associated triskelions and adaptor complexes but are devoid of membrane. In many instances, structural information on clathrin cages, coats, or coated vesicles obtained by electron microscopy and by biochemical analyses have been complementary.

Numerous visualizations of CCV by electron microscopy have shown the triskelions form a polygonal lattice (Figure 2C) (Heuser and Kirchhausen, 1985; Keen et al., 1979; Lisanti et al., 1982; Schmid et al., 1982; Vigers et al., 1986b). The faces of the lattice are a mixture of hexagons and pentagons. Although triskelions are themselves nonplanar structures the curvature of the clathrin coat is provided by the pentagonal faces: twelve pentagons being needed to coat a completely closed vesicle (Heuser and Kirchhausen, 1985). The hexagonal faces, which do not contribute to curvature, increase in numbers with the size of the coated vesicle.

Within the polymerized triskelion lattice, each vertex is occupied by one triskelion with its three legs symmetrically radiating from the vertex. Each leg spans two edges of the lattice, one by the proximal and the other by the distal leg segment.

Because of this arrangement, each edge contains two proximal and two distal leg segments. Of these, each of the two proximal and the two distal leg segments are in antiparallel orientation to each other (Keen, 1987; Kirchhausen and Harrison, 1981). This geometry also results in the presence of three terminal domains at each vertex (Figure 2C). Reconstructed images of clathrin cages in vitreous ice show that the terminal domains protrude inward from each vertex (Vigers et al. 1986b). Because clathrin LCs, which are bound to the proximal segment of the clathrin HCs in triskelions, are readily accessible to proteases and regulatory proteins within CCVs, the proximal leg segments likely reside on the cytoplasmic side of the lattice, whereas the distal segments are located beneath them (Kirchhausen and Harrison, 1981).

In CCV, the adaptor complexes AP-1 and AP-2 are located between the lattice of clathrin triskelions and the membrane (Figure 2C). There they serve as connecting links by noncovalently interacting with both the triskelions in the lattice and the cytoplasmic portions of proteins localized in the vesicle membrane. Image reconstruction of clathrin coats and CCV in vitreous ice indicated an interaction of the AP-2 complex with the triskelion lattice via the terminal domains. This result and additional evidence suggest a 1:1 stoichiometric association of AP-2 with triskelions and the presence of defined binding sites in both AP-2 and triskelions that mediate their interaction (Pearse and Robinson, 1984; Vigers et al., 1986a; Zaremba and Keen, 1983).

Two AP-2 binding sites have been defined in clathrin triskelions and clathrin cages. One site is located on either the vertex, the proximal leg segment, or the portion of the distal leg that remains after proteolytic digestion (Keen et al., 1991). This interaction is sensitive to Tris-HCl and to sodium tripolyphosphate (NaPPPi). The second site involves the terminal domain but is an interaction that may be qualitatively different in isolated triskelions and in assembled coated vesicles (Murphy and Keen, 1992). Mapping of the clathrin-binding site on the AP-2 complex produced conflicting results. Whereas Keen and Beck (1989) concluded that the AP-2 brick is sufficient for interaction with clathrin as judged by absence of release of proteolyzed AP-2 complexes from clathrin coats, Schröder and Ungewickell (1991) report that β adaptin needs to be intact to observe association with clathrin cages. In support of the first results, reconstructed images of clathrin coats frozen in ice suggest that the AP brick is in contact with the terminal domain (Vigers et al., 1986a), whereas the capability of purified β adaptin to bind to clathrin is consistent with the latter hypothesis (Ahle and Ungewickell, 1989). It seems possible that the observations by Schröder and Ungewickell (1991) are more correct because proteolyzed AP-2 bricks may have aggregated inside clathrin cages under conditions used by Keen and Beck (1989), thus preventing their release. It is unclear, however, which of the two AP-2 binding sites defined on clathrin cages is used by β adaptin or whether β adaptin can interact with both sites simultaneously. It has been proposed that the similar structure of the β and β′ adaptins in the AP-1 and AP-2 complex may reflect homologous interactions with clathrin.

The hypothesized interaction of the AP brick with clathrin in CCV suggests that the ears of the AP complex point toward the membrane. Because they are more variable in sequence among the adaptins than the brick domain, the ears were implicated in interacting with the cytoplasmic tails of integral membrane proteins. Although the involvement of the ears in binding to cytoplasmic portions of vesicle-membrane receptors has never been directly demonstrated, intact AP complexes possess this ability. AP complexes interact with affinity matrices containing immobilized-receptor tail peptides and addition of soluble tail peptides competes with immobilized tails for binding (Pearse, 1988). The AP-2 complex recognizes the 50-amino acids long cytoplasmic tail of the LDL receptor, the immunoglobulin receptor tail, and intact mannose 6-phosphate receptors (Glickman et al., 1989; Pearse, 1988). In a different experimental system that measures AP-2 binding to plasma membrane fragments derived from cells broken by freeze/thawing, intact AP-2 complexes as well as purified α adaptin, but not β adaptin, interact with the membrane suggesting that α adaptin largely mediates the interaction of AP-2 complexes with the membrane (Chang et al.,

1993). Soluble receptor tail peptides, in this case of rat hepatic lectin and polymeric immunoglobulin receptor, only partially compete with the membranes for AP-2 binding even at high molar excess of competitor, indicating that the binding of AP-2 complexes to receptor tails is a weak affinity interaction and that additional distinct high-affinity binding sites for AP-2 complexes exist in plasma membranes (Chang et al., 1993).

Analysis of a wild-type and a mutant influenza hemagglutinin indicates a tyrosine is involved in the structural motif in the receptor tails that mediate AP-2 interaction (Pearse, 1988). The wild-type influenza hemagglutinin is not internalized at the plasma membrane via receptor-mediated endocytosis, whereas the mutant hemagglutinin localizes to coated pits in the plasma membrane and is efficiently endocytosed. The mutation that enables internalization is a substitution of cysteine in the wild-type to tyrosine in the mutant. Only the mutant 8-amino acid long tail peptide containing the tyrosine residue competes with the LDL receptor tail for AP-2 binding, which suggests that the tyrosine residue is an essential part of a recognition motif for AP-2 (Pearse, 1988). Furthermore, all receptor tails that bind to AP-2 (LDL receptor, immunoglobulin receptor, the mannose 6-phosphate receptor, and rat hepatic lectin) also contain a tyrosine in their cytoplasmic portions (Chang et al., 1993; Pearse, 1988). The tyrosine containing recognition motif has been further defined to consist of the tyrosine present in a tight turn exposed in the structure of the receptor tails (Chen et al., 1990; Collawn et al., 1990; Ktistakis et al., 1990; Vaux, 1992).

Although the AP-1 complex does not bind to the LDL receptor tail, it also interacts with the mannose 6-phosphate receptor. Because neither the AP-1 nor the AP-2 complexes compete with each other for binding to the mannose 6-phosphate receptor, each adaptor complex likely recognizes a distinct and nonoverlapping receptor site. Consistent with this notion, mutation of tyrosines 1322 and 1324 in the mannose 6-phosphate receptor abolishes binding of AP-2 but has no effect on the AP-1 interaction (Glickman et al., 1989).

III. ASSEMBLY AND DISASSEMBLY

Clathrin undergoes a dynamic cycle of assembly and disassembly in the cellular cytoplasm. Because the clathrin HC and LCs have half-lives of between 24 and 50 h, whereas a cycle of assembly and disassembly takes only minutes to complete, triskelions probably undergo multiple rounds of assembly and disassembly (Acton and Brodsky, 1990; Brodsky et al., 1991). Assembly of triskelions into clathrin lattices occurs on the cytoplasmic side of membranes at a limited number of sites (Moore et al., 1987), the clathrin-coated pits. At these sites, CCV pinch off the membrane. In the cytoplasm, the clathrin coat rapidly disassembles leading to recycling of the triskelions in a form that enables them to participate in assembly of a new coat. The uncoated vesicle is then competent for fusion with a target compartment, for example, endosomes, after endocytosis at the plasma membrane. This clathrin cycle requires several regulatory steps. Futile assembly and disassembly of clathrin in the cytoplasm needs to be inhibited. Release of triskelions from lattices at coated pits needs to be prevented while uncoating of vesicles in the cytoplasm proceeds rapidly. Models that take these regulatory requirements into account will be presented and discussed.

A. Assembly

Clathrin assembly onto membranes is a multistep process. It is initiated by nucleation of coated pits on membranes and involves several distinct components, the dynamic vesicle bilayer with its integral and associated proteins, the adaptor complexes, clathrin itself, and possibly additional cytosolic factors. Knowledge of this process has been gained largely from *in vitro* studies aimed at the dissection and reconstitution of individual aspects of clathrin assembly. These include assembly of clathrin cages from triskelions and of clathrin coats from mixtures of triskelions and adaptor complexes as well as systems to study coated pit formation and coated vesicle budding.

Clathrin triskelions are the assembly units of clathrin lattices (Ungewickell and Branton, 1981).

Because one triskelion is located at each vertex of a CCV and each leg spans two edges, the contacts between triskelions are extensive. Because of the nonplanar structure of triskelions and the bend of the leg between the proximal and the distal leg segments, triskelions may not need to undergo major conformational changes during assembly (Kirchhausen et al., 1986). Moreover, the HC-HC contacts are potentially similar, irrespective of hexagon or pentagon formation (Kirchhausen and Harrison, 1981). Triskelions extracted and purified in 1 *M* Tris-HCl can assemble *in vitro* into polygonal clathrin cages. Cage formation does not require ATP and occurs in a low ionic-strength buffer containing 2 m*M* $CaCl_2$, pH ≤6.5 (Keen et al., 1979; Kirchhausen and Harrison, 1981; Winkler and Stanley, 1983). Triskelions devoid of LCs can also assemble into cages (Winkler and Stanley, 1983). Thus, the LCs are not essential for cage assembly, indicating that the bonds formed between triskelions are sufficient for assembly and formation of the curvature of the cage. In the absence of LCs, there is no Ca-requirement for assembly and cage formation can proceed under physiological conditions suggesting the LCs have a regulatory function to prevent self-association of triskelions in the cytoplasm (Ungewickell and Ungewickell, 1991).

The requirements for assembly have been defined further by investigating the assembly of triskelions obtained from clathrin cages digested with either trypsin or elastase. These treatments leave the cages assembled but result in the loss of clathrin LCs. Trypsin cleavage additionally releases the terminal domains and parts of the distal leg segments from cages. The effects of elastase treatment are less defined, but only a small part of the HC distal leg segment seems to be lost (Schmid et al., 1982; Vigers et al., 1986b). After experimental dissociation of the proteolyzed cages, trypsin-treated cages are capable of reassembly into regular cage structures, whereas elastase-treated HCs form irregular aggregates (Schmid et al., 1982). This is surprising because elastase treated HCs are presumably less degraded than those treated with trypsin. During reaggregation of the elastase-treated HCs, triskelion interactions that involve some portion of the distal leg may fail to form because of the elastase action. For example, a loose portion of the distal leg might interfere sterically with ordered cage assembly. Although such interference may abolish some HC-HC contacts, these interactions are apparently not required to mediate assembly because this part of the distal leg is probably missing in the assembly-competent HCs derived from trypsin-treated cages. Additionally, a region on the proximal leg segment of the HC within 11 nm of the vertex that overlaps with the LC binding site (see earlier) is involved in assembly. This site contains the HC epitopes of the monoclonal antibodies X19 and X35, which prevent ordered cage assembly (Blank and Brodsky, 1986, 1987; Näthke et al., 1992).

In contrast to assembly of purified triskelions into cages, their assembly into clathrin coats in the presence of adaptor complexes proceeds spontaneously under physiological conditions. Similar to assembly of triskelions alone, there is no apparent requirement for ATP (Zaremba and Keen, 1983). In comparison with clathrin cages, assembled clathrin coats tend to be more uniform in size and have an average diameter around 80 nm, which is closer to that of CCV observed *in vivo* (Pearse and Robinson, 1984). Added AP-2 complexes need to be intact structures to promote clathrin assembly. Although β adaptin is likely involved in mediating binding of the AP-2 complex to triskelions, purified β adaptin following denaturation and renaturation did not itself induce clathrin assembly (Ahle and Ungewickell, 1989). Likewise, the AP-2 brick that lacks the ears is still capable of interacting with clathrin lattices (Matsui and Kirchhausen, 1990) but has lost the ability to promote assembly (Zaremba and Keen, 1985). Although the ears themselves are not thought to contain the clathrin-binding site, the conformation of the brick might be disturbed in their absence, thereby leading to loss of assembly activity. Possibly, intact adaptor complexes are bivalent and can interact with two triskelions simultaneously (Keen, 1987). Through their specific binding interactions with triskelions, the AP-complexes might orient triskelions with respect to each other, thus facilitating their binding and assembly (Keen, 1987).

How AP-complexes achieve assembly of relatively uniform sized coats is unknown. Although the AP50 subunit can be phosphorylated *in vivo,*

phosphorylation does not affect the ability of AP-2 complexes to induce assembly (Keen et al., 1987). Various other components of the clathrin coat, such as the β′- and γ-adaptins of the AP-1 complex, the α_A-adaptin of the AP-2 complex and LCb can be phosphorylated (Morris et al., 1990). Although phosphorylation has been speculated to modulate the interactions of adaptor complexes with clathrin and the membrane (Morris et al., 1990), no direct evidence for such a regulation has yet been obtained. The coated vesicle associated kinases that are at least in part responsible for the phosphorylation of susceptible coat constituents have recently been reviewed by Schmid (1992).

In contrast to the properties *in vitro,* clathrin triskelions and adaptor complexes of intact cells do not assemble in the cytoplasm but at defined sites on the plasma membrane or the TGN. Although *in vitro* studies have provided valuable insight into the interactions between the coat constituents, assembly of adaptor complexes and clathrin triskelions on membranes *in vivo* might differ from assembly in solution. Additional complexity results from interactions with the membrane and its associated proteins. Assembly of clathrin at coated pits has to lead to the incorporation and concentration of membrane-bound receptors into the vesicle and to exclusion of others. Formation of clathrin-coated secretory granules involves specific inclusion of the molecules to be secreted into the vesicle lumen.

Different subsets of membrane-bound receptors are incorporated into coated vesicles at the cell surface and at the TGN. For example, the LDL receptor is present in Golgi membranes and the plasma membrane. Although newly synthesized LDL receptors travel from the Golgi complex to the plasma membrane with the bulk flow along the constitutive secretory pathway (independent of clathrin), however, LDL receptors localize to coated pits at the cell surface. One way to achieve this differential sorting of the LDL receptor is to postulate that the LDL receptor is able to interact with AP-2 complexes only when ligand is bound and those are available exclusively at the cell surface. However, this mechanism is not operating because LDL receptors are endocytosed irrespective of the presence or absence of their ligand. Interestingly, the AP-1 and AP-2 complexes localize to different subsets of clathrin-coated pits and clathrin-coated vesicles. The AP-2 complex is found exclusively in coated pits at the plasma membrane and on endocytic CCVs, whereas the AP-1 complex is restricted to the TGN and derivative CCVs (Ahle et al., 1988; Pearse and Robinson, 1990; Robinson, 1987; Robinson and Pearse, 1986). Therefore, sorting of membrane receptors into CCV at the TGN or the plasma membrane could be achieved by displaying recognition motifs for either AP-1 complexes, AP-2 complexes (the LDL receptor), or both complexes (the mannose-6-phosphate receptor) in the cytoplasmic receptor tail.

To achieve sorting of receptors by interaction with AP-1 and AP-2 complexes that are differentially localized, mechanisms that recruit AP-1 and AP-2 complexes to their respective membranes must exist. These mechanisms must be independent of the weak affinity interaction with receptor tails because a receptor like the mannose 6-phosphate receptor displays the recognition motifs for both AP complexes and is present in both TGN and plasma membranes. Instead, two different binding sites for AP-1 and AP-2 complexes could exist that are independent of the weak affinity interaction with receptor tails. AP-1 complexes could be localized by postulating that AP-1 binding sites are present only on TGN membranes, whereas AP-2 localizes by binding to AP-2 receptors present only in plasma membranes. Such an interaction of AP-2 with a plasma membrane receptor might be of high affinity because soluble receptor tail peptides were ineffective, even at high concentrations, to fully compete with membranes for AP-2 binding, although, alternatively, this effect may be attributed to an aberrant conformation of the peptides themselves (Chang et al., 1993). A recently identified 45-kDa protein fragment that has been released from the plasma membrane by proteolysis might be a domain of the high-affinity AP-2 receptor because it is a potent inhibitor for AP-2 binding to plasma membranes. Possibly, AP-2 binding mediated by the brick domain to a high-affinity receptor in plasma membranes might be the first step in coated-pit nucleation, followed by trapping of membrane receptors to the coated pit via the weaker receptor

tail interaction (Figure 3) (Chang et al., 1993; Peeler et al., 1993).

Evidence documenting differences between membrane association of AP-1 and AP-2 complexes that may distinguish the high-affinity AP-1 and AP-2 receptors has been accumulated recently. First, AP-2, but not AP-1, complexes are capable of directly associating with liposomes of lipid composition that resemble the plasma membrane and the α-adaptin subunit of the complex is important for the liposome interaction (Beck et al., 1992). Possibly, lipid and the 45-kDa proteolytic protein fragment may therefore contribute to the AP-2 receptor and binding to it may be mediated by α adaptin (Beck et al., 1992; Chang et al., 1993; Peeler et al., 1993). Second, AP-2, but not AP-1, complexes can self-associate under conditions that would allow coat assembly *in vitro* if clathrin were present. Although the AP-2 self-aggregation is not required for coat assembly in vitro, self-aggregation of AP-2 complexes enhances their association with the membrane, possibly their interaction with the high-affinity receptor (Beck and Keen, 1991a, b; Chang et al., 1993). Finally, AP-1, but not AP-2, association with the membrane is sensitive to the drug brefeldin A (BFA).

BFA causes mixing of normally distinct subcellular organelles such as the ER and the Golgi apparatus. The drug prevents formation of COP-coated transport vesicles that traffic between the ER and the various subcompartments of the Golgi apparatus. Instead of vesicle budding, tubular processes form that are fusion competent with their target membrane because of the absence of the coat. The fusion events lead to intermixing of the organelles (Klausner et al., 1992). The earliest observed effect of BFA is the inhibition of the binding of β-COP, a constituent of the COP coat, to budding vesicles on ER and Golgi membranes. β-COP shows sequence homology to β-adaptin (Duden et al., 1991). Interestingly, AP-1 com-

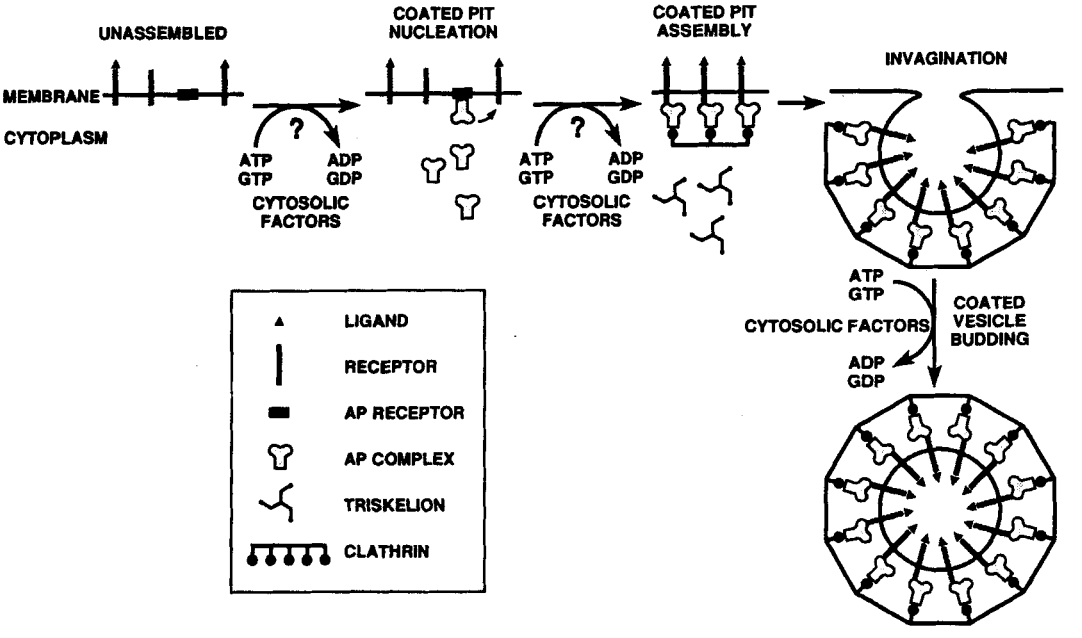


FIGURE 3. Model of clathrin-coated vesicle formation. Coated-pit formation begins by nucleation on the cytoplasmic side of the membrane by high-affinity binding of an AP complex to an AP-receptor. Clustering of receptor-ligand complexes occurs when the AP-complexes interact with the cytoplasmic tails of the receptors, and triskelions assemble on adaptor complexes as flat hexagonal lattices. Coated-pit invagination requires reorganization of the coat into hexagons and pentagons to achieve curvature. Membrane fission and formation of a closed clathrin coat lead to CCV budding from the membrane. Steps that might require ATP and GTP hydrolysis as well as cytosolic factors are indicated.

plexes are also prevented from associating with TGN membranes in the presence of BFA, whereas this drug has no effect on the membrane association of AP-2 complexes (Robinson and Kreis, 1992; Wong and Brodsky, 1992). In conclusion, AP-1 complexes may have a mechanism of recruitment to the membrane similar to the mechanism used by β-COP but different to AP-2 complexes. At present, it is unclear which subunit of the AP-1 complex is functionally affected by BFA action and responsible for the prevention of membrane association. The β′-adaptin of the AP-1 complex could be a preferred candidate because β-COP is more related to β-adaptin than to the α- and γ-adaptins (Duden et al., 1991). BFA does not act directly on β-COP. More likely, BFA has an effect on an ADP-ribosylation factor (ARF), which is a member of a family of monomeric GTPases that are required for the association of COPs with Golgi membranes. This reaction, in turn, is regulated by heterotrimeric GTPases (Klausner et al., 1992; Sztul et al., 1992). Whether ARF and heterotrimeric GTPases are also involved in regulating AP-1 association with TGN membranes is unknown but ARF has been identified as a constituent of CCV (Lenhard et al., 1992).

Coated pit assembly by AP-2 complexes and clathrin triskelions (Figure 3) has been studied and reconstituted in semiintact cells and plasma membranes derived from broken cells bound to a plastic surface so that their cytoplasmic side is accessible to experimental manipulation. These systems allowed examination of the requirements for adaptor and clathrin assembly at coated pits as well as vesicle budding and fission. As in intact cells (Anderson et al., 1977), these systems contain a limited number of coated pits at defined sites. The membrane can be stripped of clathrin by a high-pH buffer or of clathrin and the AP-2 complexes by high-salt conditions (Mahaffey et al., 1989, 1990; Moore et al., 1987). Clathrin triskelions rebind to high-pH stripped membranes with high affinity and in a saturable fashion. Triskelions do not reassociate, however, to membranes stripped with high salt, even in the presence of rebound AP-2 (Mahaffey et al., 1990). This result suggests the AP-2 complexes are present in an inactive form and must be reactivated before clathrin can rebind, possibly by an uncharacterized cytosolic factor. Although binding of purified AP-2 complexes and clathrin in these assays seems to be independent of ATP and ATP hydrolysis, *de novo* nucleation of coated pits and the budding of coated vesicles off the membrane recently has been shown to be dependent on cytosol and ATP. ATP and cytosol might be required for reactivation of AP-2 complexes, after or during their removal from endocytic vesicles, to restore their ability to bind to membranes and initiate coated-pit formation (Schmid and Smythe, 1991; Smythe et al., 1989). Heterotrimeric GTP-binding proteins are candidate components of cytosol participating in coated-pit assembly and CCV budding as these processes are inhibited by AlF_4–, GTPγS, and mastoparan (Carter et al., 1993; Leyte et al., 1992; Schmid, 1992; Smythe et al., 1992; Tooze et al., 1990b). However, no GTP-binding protein has yet been isolated from cytosol and been directly shown to be a constitutive participant or a regulatory factor in these processes. Another GTP-binding protein implicated to function in endocytosis is dynamin, which is the mammalian homologue of the protein responsible for the *shibire* mutation in *Drosophila* (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). Flies carrying this mutation exhibit a temperature-sensitive block in endocytosis causing the accumulation of coated pits presumably because membrane fission or CCV budding cannot be completed (Kosaka and Ikeda, 1983). As for other GTP-binding proteins, direct biochemical evidence for a function of dynamin in the formation of CCV is lacking.

AP-2 complexes that interact with receptor tails of membrane proteins after recruitment to the membrane could exclude other membrane proteins from coated pits (Bretscher et al., 1980) by self-association with other AP-2 complexes (Beck and Keen, 1991a) or by association with clathrin. If each AP-2 complex at a coated pit interacts with one receptor tail, the resulting close packaging might automatically exclude unbound proteins from the coated pit (Pearse and Robinson, 1984).

B. Disassembly

Clathrin disassembly from CCV *in vitro* can be enzymatically catalyzed by the cytosolic heat shock cognate protein Hsc70 (Chappell et al., 1986; Schmid et al., 1984; Ungewickell, 1985), which acts as a clathrin-dependent ATPase that removes triskelions from the coat. During uncoating, the adaptor complexes stay bound to the integral membrane receptors of the vesicle (Heuser and Keen, 1988).

Hsc70 is a constitutively expressed member of the 70-kDa family of stress-induced proteins, members of which are present in all organisms. In prokaryotes such as *E. coli*, which do not possess clathrin, the major homolog to the mammalian Hsc70 is DnaK. Although 48% identical to Hsc70 in amino acid sequence, DnaK does not possess uncoating activity. In contrast, yeast members of the 70-kDa heat shock protein family possess uncoating activity (Rothman and Schmid, 1986).

Assays of CCV uncoating *in vitro* have used clathrin-coated vesicles or clathrin cages as substrates (Patzer et al., 1982). Because cages are composed only of triskelions, this observation demonstrated that Hsc70 acts directly on the triskelions but not on adaptor complexes or the vesicle membrane.

Hsc70 interacts with CCVs stoichiometrically. In cages but not in purified triskelions, each triskelion binds three Hsc70 molecules, likely one at each leg (Heuser and Steer, 1989; Rothman and Schmid, 1986). An average of three molecules of ATP are hydrolyzed for the removal of one triskelion and, therefore, removal of each leg is thought to require hydrolysis of one ATP molecule (Rothman and Schmid, 1986). When all three legs of one triskelion are freed, the triskelion is released from the coat into the cytoplasm (Schmid and Rothman, 1985a). The five-step model for clathrin uncoating (Figure 4) presented below, which is adapted and modified from DeLuca-Flaherty et al. (1990) and Heuser and Steer (1989), attempts to synthesize the many experimental observations and discusses them in the context of each step.

1. Hsc70 binds stoichiometrically to triskelions assembled in clathrin-coated vesicles in its ATP-bound form. Binding does not occur to free triskelions. Two different binding sites have been identified for Hsc70 on assembled clathrin. One binding site is present on clathrin LC (Schmid et al., 1984; Schmid and Rothman, 1985b) and has been localized to residues 47–71 of the LC sequence (DeLuca-Flaherty et al., 1990). The other binding site involves the HC terminal domains. Hsc70 might initially interact with the terminal domains (Heuser and Steer, 1989), because cages that contain trypsinized triskelions lacking the terminal domain but possessing intact LCs do not bind Hsc70 (Rothman and Schmid, 1986). AP-2 complexes interact with the terminal domain in clathrin coats (Vigers et al., 1986a). Binding of Hsc70 to the terminal domains might displace the AP-2 complexes, leading to detachment of the clathrin lattice and the vesicle. Thus, the AP complexes are retained on the vesicle (Heuser and Keen, 1988).
2. After three Hsc70-ATP complexes are bound to the terminal domains located at one vertex, the Hsc70-ATP molecules could shift to interact with the LC-binding site. It has been suggested that clathrin uncoating is a cooperative process as three Hsc70 molecules can dissociate one clathrin triskelion, even at low ratios of Hsc70 to clathrin (Greene and Eisenberg, 1990). Hsc70 molecules could cooperatively bind to the three terminal domains localized in close proximity at one vertex. Although these three terminal domains belong to three different triskelions, the shift to the LCs will combine the three Hsc70 molecules on one triskelion and concentrate Hsc70 action toward triskelion removal from the coat.

Exposure of the LC-binding site for Hsc70 is promoted by higher than physiological Ca^{2+} concentrations (DeLuca-Flaherty et al., 1990). Because the Ca-binding site of the LCs is located next to the Hsc70-binding site in the LCa molecule, Ca^{2+} could exert their effect by binding to this site (Brodsky et al., 1991). A microenvironment containing as much as 0.1 *M* Ca^{2+} concentration might be created around a coated vesicle

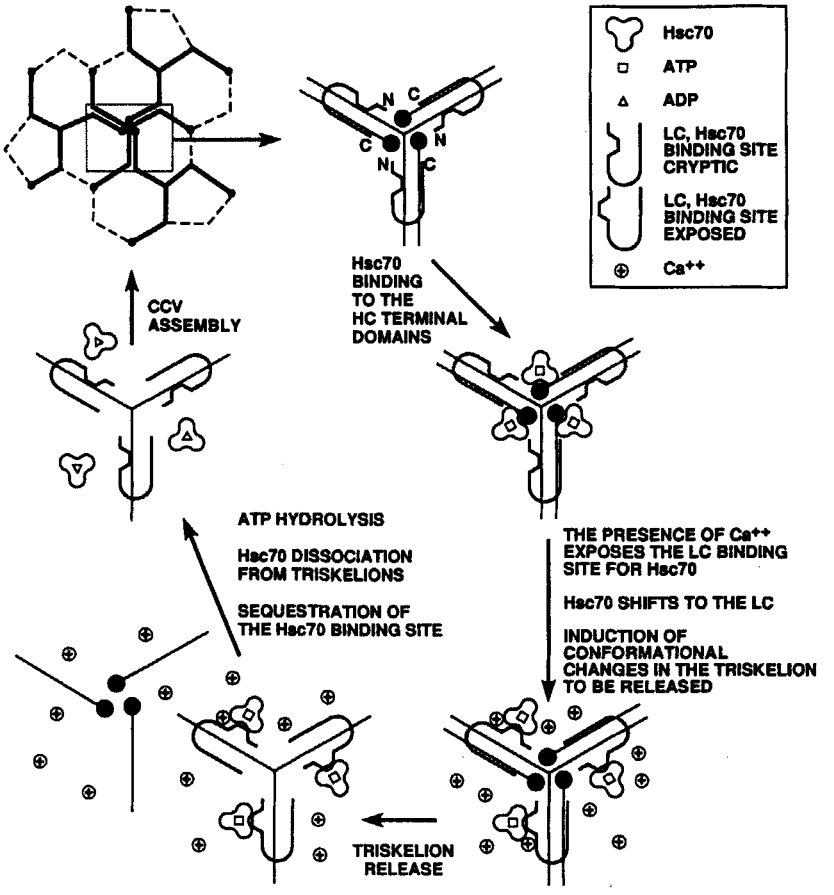


FIGURE 4. Model of clathrin-coated vesicle uncoating. At the top of the figure, a part of a clathrin lattice depicting several triskelions is shown. Blow-up: One triskelion with its proximal leg segments and associated LCs as well as three distal leg segments and their terminal domains, which belong to triskelions at the next but one vertices.

because of efflux of Ca^{2+} across the vesicle membrane from the vesicle interior, which resembles the extracellular fluid in its high Ca-content (DeLuca-Flaherty et al., 1990). Both LCa and LCb are capable of interacting with Hsc70, but LCa might be more important for interaction with Hsc70 than LCb (DeLuca-Flaherty et al., 1990). Thus, triskelions that have three LCa molecules bound might be more effective in capturing the Hsc70 molecules from the terminal domains and may be released faster from CCV than triskelions comprising mixtures of LCa and LCb or exclusively LCb. This might explain the initial burst of uncoating seen in assays followed by a second slower steady-state phase of removal (Greene and Eisenberg, 1990).

3. Binding of Hsc70-ATP to the LC-binding site and the concomitant conformational change in the LC molecules might lead to triskelion release. The presence of bound Hsc70 and the altered LC conformation might affect the conformation of other regions in the LC molecule, especially the HC-binding domain located toward the C-terminus of the Hsc70-binding site or the conserved region between LCa and LCb (residues 23–44) located toward the N-terminus. These effects might have consequences for the HC-LC interaction during uncoating, perhaps leading to perturbation

of triskelion-triskelion interactions. The HC epitopes recognized by the monoclonal antibodies X19 and X35 identify a site involved in the triskelion assembly that coincides with the clathrin LC-binding site on the HC, and X19 binding can cause disassembly of cages (Blank and Brodsky, 1986, 1987; Näthke et al., 1992). It is therefore conceivable that disturbances in this region caused by Hsc70 binding to the LC could lead to loss of triskelion-triskelion interactions and ultimately to triskelion release.

4. In Step 3, triskelions are released as complexes with three Hsc70-ATP molecules. Although released triskelions have been experimentally observed as complexes with three Hsc70 molecules bound near the vertex (Heuser and Steer, 1989; Schlossman et al., 1984), it has not been demonstrated directly whether ATP has been hydrolyzed at this stage. However, ATPγS can substitute for ATP in the release of triskelions from CCV, indicating that ATP hydrolysis is not required for triskelion release (Heuser and Steer, 1989; Schlossman et al., 1984).
5. ATP is hydrolyzed to release the uncoating enzyme Hsc70 from triskelions after dissociation from the coat. This hypothesis is consistent with the mechanism by which Hsc70 releases bound peptide substrates (Flynn et al., 1989).

The clathrin cycle exhibits several regulatory features. Clathrin and adaptor complexes are present in the cytoplasm in sufficient concentrations and under conditions that drive coat assembly *in vitro*. In cells, however, clathrin assembles onto membranes only and no assembly leading to membrane-free coats in the cytoplasm is observed. Triskelions in complexes with Hsc70 cannot reassemble into cages *in vitro* (Schlossman et al., 1984). Clathrin might therefore be present in an inactive state in the cytoplasm, reactivating at the membrane by ATP hydrolysis and Hsc70 release just before their reincorporation into the expanding lattice of a coated pit. ATP depletion in the cytoplasm leads to assembly of coats in the cytoplasm that might be mediated by triskelions released from Hsc70 and becoming assembly competent. An alternative to this mechanism is for cytoplasmic triskelions to be assembly competent but that Hsc70 acts to dissociate nascent polymers before larger assemblies such as coats can form. For this latter hypothesis to be correct, however, it would be necessary to invoke that Hsc70 can interact with triskelions, even in the absence of elevated Ca^{2+} levels.

Clathrin triskelions assembled onto coated pits are immune to the uncoating action of Hsc70 (Heuser and Steer, 1989). Although Ca-ATPase present in the plasma membrane might be excluded from clathrin-coated pits, nearby present Ca-ATPase might reduce Ca^{2+} levels on the cytosolic side of coated pits, thus preventing release of triskelions from clathrin-coated pits (DeLuca-Flaherty et al., 1990). Alternatively, it has been proposed that the clathrin lattice of coated pits consists exclusively of hexagonal faces and that the coat reorganizes during vesicle budding to create the pentagons necessary for vesicle curvature. It has been reported that adaptor complexes in reconstituted clathrin coats were found to interact with the HC terminal domains predominantly at hexagons and less at pentagons. Therefore, it might be possible that in coated pits, the terminal domains interact with adaptor complexes and are not available for interaction with Hsc70. This would prevent clathrin disassembly at the coated pit.

After clathrin disassembly, the uncoated vesicles fuse with a target membrane such as endosomal membranes in the case of receptor-mediated endocytosis or the plasma membrane in the case of regulated secretion. The adaptor complex AP-2 stays bound to endocytosed vesicles after uncoating and might play a role in targeting the vesicle to the fusion compartment, the endosome. AP-2 complexes present on the membrane cause aggregation with other AP-2-bound membranous structures such as preexisting endosomes or other uncoated vesicles. The α adaptin may mediate this aggregation, which was not observed with the AP-1 complex or the AP180 polypeptide. Therefore, uncoated secretory vesicles seem to have a different mode of targeting to the plasma membrane than endocytic vesicles (Beck et al., 1992).

IV. CLATHRIN IN NEURONS

A. Function of Clathrin-Coated Vesicles in Neurons

Neurons of the peripheral and the central nervous systems are polarized secretory cells with distinct morphology that create an elaborate cell/cell communication network. The neuronal cell body — the perikaryon — contains the nucleus, other membrane-bound organelles, including mitochondria, ER, and Golgi apparatus, and the majority of cytoplasmic ribosomes. Therefore, the perikaryon is the site of almost all biosynthetic activity. Synaptic vesicles loaded with neurotransmitters constitute the secretory vesicles of neurons. They are transported along the nerve axon to the nerve terminal, which is the site of exocytosis. Thus, in comparison with other secretory cells, the site of exocytosis in neurons is far from the site of secretory vesicle biogenesis. Neurotransmitter release, by fusion of synaptic vesicles with the presynaptic membrane, is a regulated process that occurs on stimulation of nerve cells and involves a transient 10- to 100-fold elevation of the cytoplasmic Ca^{2+}-concentration in the nerve terminal to give a final concentration of about 10 μM. This rise in Ca^{2+} leads to release of synapsin I (a peripheral secretory vesicle protein) from the synaptic vesicles, thus allowing them to fuse with the presynaptic membrane. Depending on its identity, the released neurotransmitter is either degraded enzymatically in the synaptic cleft or transported back into the presynaptic cytoplasm by active transport. Synaptic membranes containing synaptic vesicle proteins are endocytosed to compensate for the increase in plasma membrane created by synaptic vesicle fusion. On endosomes in the nerve terminal, the recycled synaptic vesicle proteins and membrane reform vesicles, which are subsequently refilled with neurotransmitter by active transport. On a new stimulus, the synaptic vesicles can undergo a new round of release (Kelly, 1991).

Clathrin-coated vesicles appear to fulfill several distinct functions in neurons. Most commonly proposed is a role for CCV in the recycling of presynaptic membrane after fusion of synaptic vesicles and neurotransmitter release. Studies to investigate recycling of synaptic membranes have used electron microscopy to visualize the nerve terminal and found numerous coated pits and coated vesicles on the presynaptic membrane and in the presynaptic cytoplasm (for a review see Heuser, 1989). But because the number of coated vesicles present in the nerve terminals did not correlate with the intensity of nerve stimulation, it was doubted that CCV provide the only mechanism for recycling synaptic membranes. Accordingly, an additional mechanism in which synaptic membranes are reformed without coated intermediates was proposed.

These morphological studies did not reveal whether CCV reform directly at sites of synaptic vesicle fusion (the direct model) or whether CCV-mediated membrane recycling occurs at zones spatially separated from the active zones of synaptic vesicle exocytosis (the indirect model) (Figure 5). In the direct model, synaptic vesicle-specific proteins are internalized before they diffuse away from the site of fusion. In contrast, the indirect model postulates that synaptic vesicle proteins intermingle with components of the presynaptic plasma membrane and thus must be sorted and concentrated into coated pits. The latter model is reminiscent of the concentration and internalization of receptor-ligand complexes occurring at the plasma membrane of other cell types (Heuser, 1989).

Recently, CCV purified from nerve terminals have been shown to contain several membrane proteins characteristic of synaptic vesicles, including synaptophysin, synaptotagmin, p29, synaptobrevin, and the 116-kDa subunit of the synaptic vesicle-associated proton pump. Furthermore, CCV prepared from whole brain homogenates were similar in composition to CCV purified from preparations of isolated nerve terminals. Although this result cannot distinguish the direct and the indirect models of synaptic vesicle membrane recycling, it indicates that a predominant role of brain CCV is in synaptic-vesicle recycling (Maycox et al., 1992) and perhaps other endocytic processes at the nerve terminal.

In addition to their functions at the nerve terminal, CCV form at the TGN in the neuronal

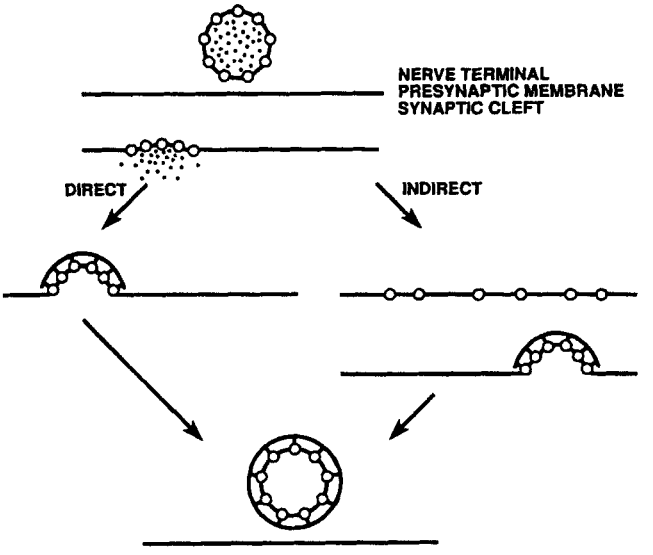


FIGURE 5. The direct and the indirect models for synaptic-vesicle recycling. Fusion of a synaptic vesicle with the presynaptic membrane releases neurotransmitter (stippled) into the synaptic cleft. In the direct model (left side of the diagram), the membrane and synaptic-vesicle proteins (o) are retrieved via CCV formation at the site of neurotransmitter release. In the indirect model (right side of the diagram), synaptic-vesicle proteins diffuse in the membrane. They are reconcentrated into coated pits and internalized at a site spatially separated from the site of neurotransmitter release.

perikaryon and may represent the class of vesicles termed large density-core vesicles (LDCV). LDCV carry neuromodulators and proteins that are secreted at the nerve terminal in response to a physiological stimulus. They resemble both the secretory granules of endocrine cells and the secretory vesicles of other cell types that possess a regulatory secretory pathway in that the transported material appears in electron micrographs to be condensed and aggregated (De Camilli and Jahn, 1990). Although the LDCV are uncoated prior to their fast transport along the cell axon, the clathrin coat might be involved in initiating the transport (Stone et al., 1984). Clathrin itself is not associated with fast axonal transport but is transported as triskelions with SCb, one component of slow axonal transport (Garner and Lasek, 1981; Gower and Tytell, 1987).

Both LDCV and synaptic vesicles form at the TGN but differ in that synaptic vesicles do not possess a high-density core and may not pinch off from the TGN as CCV during biogenesis. Thus, the clathrin coat may not be involved in concentrating neurotransmitters into newly forming synaptic vesicles. Instead, this may occur by active transport across the synaptic-vesicle membrane as in nerve terminals (Kelly, 1991).

CCV are also found in dendrites that protrude from the perikaryon. These CCV are probably equivalent to plasma membrane-derived endocytic vesicles of other cell types and are involved in endocytic processes such as transferrin uptake.

B. Neuron-Specific Modifications of CCV

Several components of the clathrin coat in neurons exhibit either neuron-specific modifications or are specifically associated with CCV in neurons. Each of these modifications may represent adaptations to either the specialized functions of CCV in neurons or the unusual morphology of these cells.

1. Clathrin Light Chains LCa and LCb

Neurons of all mammals investigated as well as chicken express neuron-specific isoforms of LCa and LCb (Wong et al., 1990). The neuron-specific forms of LCa and LCb are absent from peripheral tissues and other brain cells (collectively referred to as glial cells) (Wong et al., 1990). In mammalian neurons, two neuron-specific isoforms of LCa and one neuron-specific isoform of LCb can be distinguished (Wong et al., 1990) that exhibit a reduced mobility on SDS-PAGE by 2–4 kDa in comparison with the peripheral LC isoforms (Brodsky and Parham, 1983; Creutz and Harrison, 1984; Holmes et al., 1984; Pearse, 1978). cDNA sequences encoding neuron-specific LCb contain an insertion coding for 18 amino acids located approximately two thirds toward the C-terminus on the carboxy-terminal side of the clathrin HC-binding domain. Two types of LCa cDNA clones were found, one type containing a homologous 18 amino acid insertion and one type with a 30 amino acid insertion at the same site (Jackson et al., 1987; Kirchhausen et al., 1987b). In the two types of neuron-specific LCa clones, the 18 amino acid insertion corresponds to the N-terminal part of the 30 amino acid insertion (Jackson et al., 1987; Kirchhausen et al., 1987b). Analysis of rat genomic DNA of LCb reveals that the 18 amino acid insertion is encoded on a separate exon (Stamm et al., 1992). In human LCa, the 30 amino acid neuron-specific insert is encoded by two exons, one coding for the N-terminal 18 amino acids homologous to LCb and a separate exon encoding for the additional 12 amino acids unique to LCa (unpublished results).

Whereas LCa and LCb are encoded by separate genes, the peripheral and neuron-specific forms of both LCa and LCb arise by alternative splicing (Jackson et al., 1987; Kirchhausen et al., 1987b). Except for two point substitutions that could reflect allelic polymorphisms among different individuals of a species, the 5′ and 3′ untranslated regions and the homologous coding regions of neuronal and peripheral forms have identical sequences. This strongly argues that peripheral and neuron-specific LCs are transcribed from a single gene and that they arise by neuron-specific splicing (Jackson et al., 1987; Kirchhausen et al., 1987b; Stamm et al., 1992). Because the neuron-specific sequences are encoded by separate exons, the peripheral forms of LCa and LCb most likely arise by exon skipping. Neurons express a neuron-specific *trans*-acting factor that is part of the spliceosome and implicated in the formation of neuron-specific isoforms by alternative mRNA splicing (Schmauss et al., 1992). Because both LCa and LCb possess homologous neuron-specific inserts, the 18 amino acid length insertion sequences likely evolved before duplication of the LCa and LCb genes. Possibly, this sequence already existed before the divergence of yeast and mammalian LCs because amino acid residues 218–238 of the yeast LC are reminiscent of the LCb neuron-specific sequence (Silveira et al., 1990). Presence of the additional 12-amino acids long sequence in LCa but not LCb indicates that this segment was incorporated into the LCa gene after duplication of the ancestor common to LCa and LCb. All mammalian clathrin LC sequences characterized (human, cow, and rat) contain the 30 amino acid insertions in LCa and their sequences are absolutely conserved among species (Jackson and Parham, 1988). Interestingly, in this context, chicken only seem to express one higher molecular weight form of LCa in brain tissue (Wong et al., 1990). The SDS-PAGE electrophoretic difference in mobility of these two forms seems more likely to correspond to an 18 amino acid difference in size rather than 30 amino acids.

Although LCa and LCb exhibit functional diversification regarding several segments of the protein, the 18 amino acid neuronal-specific inserts have undergone only moderate sequence divergence. Thus, these inserts may have a similar function in LCa and LCb. In contrast, the additional 12 amino acid insert unique to LCa is only expressed in conjunction with the 18-amino acid long insert, indicating that in LCa, this additional sequence might modify the neuron-specific function of the insert.

2. Assembly Polypeptide Complex AP-2

Among the components of the AP-1 and AP-2 complexes, only the 100–112-kDa α and β

polypeptides of the AP-2 complex show brain-specific modifications suggesting a neuron-specific function in CCV at the plasma membrane and not at the TGN.

Two subtypes of the 100-kDa α chain were immunologically identified and termed α_A and α_C (Robinson, 1987). The α_A and α_C proteins are encoded by related but different genes. At the protein level, the sequences are highly conserved (84% identity, with many of the differences being conservative in nature) (Robinson, 1989). α_C Chains are present in all tissues, whereas two isoforms of α_A exist that exhibit tissue-specific expression (C.L. Ball and M.S. Robinson, personal communication). One isoform of α_A is of higher molecular weight than α_C because of an insert of 41 amino acids, which has no counterpart in α_C (Robinson, 1989) and is found in the brain. In the second isoform, 22 amino acids of the 41 residue insert are missing, probably because the mRNA is differentially spliced. This α_A isoform is present in all tissues examined, including brain and its mobility in SDS-PAGE is similar to α_C (C.L. Ball and M.S. Robinson, personal communication). The inserts are proline rich and located in the stalk region of the adaptor complex, a region believed to provide a flexible hinge. Such a hinge might link the adaptor complex domains that interact with clathrin and the membrane-bound receptors, respectively. Additionally, α_A and α_C chains are more variable in their C-terminal domains, the domain that has been proposed to interact with the cytoplasmic tails of receptors. As a result of these differences, AP-2 complexes containing either α_A or α_C might select distinct receptors for internalization (Kirchhausen et al., 1989).

For the β-adaptin of the AP-2 complex, the situation is reminiscent of that for the α chains. As for the α_A chain, β-adaptin can contain a brain-specific insertion sequence of 14 amino acids that is glycine rich and located within the proline/glycine-rich stalk region. In contrast to the α chain, the two β-adaptin isoforms are the products of a single gene and arise by tissue-specific splicing, as is the case for the neuron-specific isoforms of LCa and LCb (Ponnambalam et al., 1990).

3. AP-3, AP180, and NP185

AP-3, AP180, and NP185 are the names given to acidic polypeptides of apparent molecular weights 155–185 kDa in SDS-PAGE that have been independently identified from preparations of brain CCV in three laboratories (Ahle and Ungewickell, 1986; Keen and Black, 1986; Kohtz and Puszkin, 1988). In contrast, estimates from gel filtration gave values of about 120 kDa as the molecular weight of AP180 and NP185 (Prasad and Lippoldt, 1988) and molecular cloning of AP180 established a calculated molecular weight of 91 kDa (Morris et al., 1993), Because of cross-reactivity of monoclonal antibodies with AP-3, AP180, and NP185 and their identical migration patterns in two-dimensional gels, it has been concluded that AP-3, AP180, and NP185 are identical (Murphy et al., 1991). This protein will subsequently be referred to as AP-3.

Neuron-specific AP-3 expression has been confirmed performing Northern analysis to detect mRNA expression in various tissues (Morris et al., 1993) and using a specific monoclonal antibody. Immunofluorescence microscopy revealed a punctate staining pattern in the processes of neuronal cell lines but an absence of immunoreactivity in cell lines derived from glial cells or sources of peripheral tissues (Kohtz and Puszkin, 1988). The punctate staining pattern supports an association with CCV. Interaction with clathrin has also been demonstrated *in vitro*. Both the intact AP-3 polypeptide and a 30-kDa tryptic N-terminal fragment thereof were specifically retained on a clathrin-triskelion affinity resin and eluted in the presence of increasing concentrations of Tris-HCl, conditions that also release the adaptor complexes AP-1 and AP-2 from CCV (Morris et al., 1993; Murphy et al., 1991). Similar to AP-1 and AP-2 (Keen, 1987; Keen et al., 1979) AP-3 promotes assembly of clathrin into coats of relatively homogeneous size distribution under conditions that do not induce spontaneous assembly of clathrin cages (Ahle and Ungewickell, 1986). Clathrin cages will spontaneously form in the presence of millimolar Ca^{2+} concentrations; however, cages thus formed are heterogeneous in size (Keen et al., 1979). When specific coat assembly activity is analyzed using mixtures of

AP-1, AP-2 complexes, and AP-3, AP-3 is about four times more active in promoting clathrin assembly than AP-1 and AP-2 (Lindner and Ungewickell, 1992).

In reassembled clathrin coats the stoichiometry of AP-3 and triskelions is 1:1 (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988). The same stoichiometry of interaction has been reported for AP-2 (Keen, 1987). This suggests AP-2 and AP-3 interact similarly with clathrin triskelions, a possibility that has been investigated by mapping the sites of AP-2 and AP-3 binding on clathrin triskelions. As for AP-2, interaction of AP-3 with triskelions is independent of the presence of clathrin LCs (Ahle and Ungewickell, 1986; Murphy and Keen, 1992). However, the clathrin HC needs to be intact for AP-3 to interact, whereas AP-2, which can interact with two distinct sites on clathrin, can also bind to isolated, immobilized terminal domains and cages that are devoid of the terminal globular domains and most of the distal portion of the triskelion legs (Murphy and Keen, 1992). Although the results indicate that the interactions of AP-2 complexes and AP-3 with the clathrin HC are not homologous, the binding sites may overlap because partial competition of binding to Sepharose-immobilized clathrin was observed for a mixture of AP-2 complexes and AP-3 (Murphy and Keen, 1992). Whereas AP-1 and AP-2 complexes interact with the cytoplasmic tails of receptors internalized into or transported by CCV (Glickman et al., 1989; Pearse, 1988), such an interaction has not been established for AP-3. Furthermore, whereas AP-3 extracts from CCV as a monomer, AP-1 and AP-2 are complexes composed of several different polypeptides. Although comparable in molecular weight the AP-3 polypeptide does not exhibit sequence similarity to the adaptins of the AP-1 and AP-2 complexes (Morris et al., 1993). It is therefore unknown whether AP-3 can fulfill a function as an assembly polypeptide similar to AP-1 and AP-2 *in vivo*. One report also demonstrates association of AP-3 with the 100-kDa and 50-kDa AP complexes in *in vitro* coimmunoprecipitations using an AP-3 specific monoclonal antibody (Kohtz and Puszkin, 1988).

Besides binding to the clathrin HC, AP-3 interacts with tubulin in CCV (Kohtz and Puszkin, 1989). Tubulin is a heterodimeric protein composed of α- and β-tubulin that can polymerize to form microtubules. Microtubules are an abundant cytoskeletal structure in neurons. α- and β-Tubulin are intrinsic components of CCV as judged by coimmunoprecipitation and coelution on gel filtration (Kelly et al., 1983; Pfeffer et al., 1983). It has been suggested that tubulin may link CCV to the cytoskeleton facilitating movement of CCV within the cytoplasm (Pfeffer et al., 1983). Although the exact mode of association of AP-3 and tubulin in CCV is unknown, their *in vitro* interaction seems to be enhanced by phosphorylation of tubulin by a CCV-associated casein kinase II, an activity that might reside within AP-3 itself (Kohtz and Puszkin, 1989). AP-3 has also been reported to be phosphorylated *in vivo* (Keen and Black, 1986), although the physiological relevance of this effect remains elusive.

4. Auxilin

Auxilin is an 86-kDa polypeptide that is associated with CCV from brain but not other tissues. Its neuron-specific expression, however, has not been unequivocally demonstrated (Ahle and Ungewickell, 1990). Auxilin promotes assembly of clathrin *in vitro* and becomes stoichiometrically associated with clathrin coats at a ratio of 1 molecule auxilin per clathrin HC. In comparison with AP-3 the size distribution of coats formed (50–100 nm in diameter) is broader. As for AP-3, auxilin is extracted from the clathrin coats by 0.5 *M* Tris-buffer as monomers. This questions the capacity of auxilin to function as a bivalent adaptor, for example, the AP-2 complex, although it tends to self-aggregate under physiological conditions, a property also attributed to AP-2 complexes (Beck and Keen, 1991b). When the assembly activity of various assembly polypeptides (AP-1, AP-2, AP-3, and auxilin) was analyzed *in vitro*, auxilin is as active as the AP-1 and AP-2 complexes (Lindner and Ungewickell, 1992). Association of auxilin with receptor tails has not been demonstrated.

5. p140

A protein of molecular weight 140 kDa that by peptide mapping is unrelated to AP-3 has been identified recently in coated-vesicle preparations from bovine brain. The ability of p140 to promote clathrin assembly into cages has yet to be tested (Lindner and Ungewickell, 1992).

C. Function of Brain-Specific CCV Components in Neurons

The expression of neuron-specific CCV components likely represents adaptations to neuron-specific functions and to the conditions imposed on clathrin and CCV by the specialized organization of neuronal cells. In particular, expression of neuron-specific components that mediate neuron-specific functions of CCV are expected to be developmentally controlled. That is, their expression might be induced during differentiation of neuronal cells from undifferentiated precursor cells. One line of evidence in favor of this hypothesis derived from experiments investigating the expression of AP-3 in PC12 cells (Kohtz and Puszkin, 1988). PC12 cells are derived from rat adrenal pheochromocytoma cells that differentiate into neuronal-like cells instead of chromaffin cells in the presence of NGF, as judged by outgrowth of neurites (Greene and Tischler, 1976). In the absence of NGF, PC12 cells do not express AP-3. After addition of NGF, AP-3 is expressed and distributed, as assessed by immunofluorescence microscopy, in a punctate manner in the developing neurites and growth cones (Kohtz and Puszkin, 1988). A similar developmentally controlled expression and occurrence of neuron-specific splicing events has been reported for LCb (Stamm et al., 1992).

The adaptation of CCV for neuron-specific functions might only extend to a subset of the CCV present in neurons. Some of the neuron-specific functions might be related to their specialized role in synaptic-vesicle recycling. For example, immunohistochemical data indicate that the neuron-specific LC isoforms concentrate in axon terminals (Wong et al., 1990) and AP-3 is distributed throughout the neurites in rediffer-entiated PC12 cells (Kohtz and Puszkin, 1988). In the perikaryon and in dendrites, a distinct subset of CCV might be involved in receptor-mediated endocytosis (e.g., of transferrin [Parton et al., 1992]) at the plasma membrane, processes that also exist in cells other than neurons and that, therefore, might not be expected to require specific modifications of CCV in neurons.

Neuron-specific components of adaptor complexes associated with CCV in the nerve terminal might recognize internalization signals on synaptic-vesicle components. In peripheral tissues, internalization of receptor-ligand complexes is mediated by binding of adaptor complexes to cytoplasmic receptor tails (Glickman et al., 1989; Pearse, 1988). In these receptor tails, a tyrosine residue is part of an endocytic signal. Possibly, integral synaptic-vesicle proteins may contain a different internalization motif. The brain-specific assembly polypeptides AP-3, auxilin, or the brain specific AP-2 adaptors could recognize internalization signals on synaptic vesicle components and be involved in initiating coated-vesicle assembly. Alternatively, after uncoating, the brain-specific assembly, polypeptides might deliver the recycled synaptic membranes to the appropriate fusion compartment, for example, endosomes, in the nerve terminal. In this process, brain-specific AP could directly contact the membrane of the fusion compartment or they could mediate movement of the endocytic vesicles along cytoskeletal elements, for example, microtubules, to the fusion compartment.

Neurons are unique in that the cell body, the site of new protein synthesis and vesicle biogenesis at the Golgi, are spatially separated by the axon from the nerve terminal, the site of synaptic-vesicle exocytosis and endocytosis. This requires that the complete machinery needed for endocytosis must be transported into the nerve terminals. Three major transport complexes, the fast axonal transport and the slow axonal transport components SCa and SCb have been distinguished in axons. All are thought to be based on association with particular cytoskeletal components present in the axon (Tytell et al., 1981). Although vesicles (both anterograde and retrograde) are always transported via the fast axonal transport, the components and regulators of clathrin examined so far

are all transported in the slow component b. These components include the clathrin HC itself (Garner and Lasek, 1981), the clathrin LCs (Gower and Tytell, 1987), the uncoating ATPase Hsc70 (De Waegh and Brady, 1989), and the 50-kDa component of AP-2 (Chestnut et al., 1986). Additionally, calmodulin, a mediator of calcium-regulated processes, which can interact with clathrin (Linden et al., 1981), is also transported via SCb (Tytell et al., 1981).

The neuron-specific modifications of CCV components could serve to target these molecules to the SCb axonal transport and interact with SCb-associated cytoskeletal elements. Clathrin is transported as triskelions and neither coats nor cages have been observed in axons. During the transport, the triskelions are in stable complexes with Hsc70, which may maintain clathrin disassembled (Black et al., 1991; De Waegh and Brady, 1989). Proteins in the SCb component move at a rate of about 2 mm/d. Because axons (e.g., of retinal ganglion cells) can be millimeters to several centimeters in length, transport of clathrin from the cell body to the nerve terminal may take several days on average. How does the half-life of clathrin inside cells compare with the slow transport kinetics? For a transformed B-lymphoid cell line that expresses the peripheral LC forms, the protein half-lives for clathrin heavy and light chains LCa and LCb are 50 h, 24 h, and 45 h, respectively (Acton and Brodsky, 1990). In neuronal cells, the brain-specific inserts might act to stabilize LCa and LCb, thus increasing the number of endocytic cycles triskelions can undergo after they reach the nerve terminal. The ability of the high-molecular-weight brain-specific form of LCa (30 amino acid insert) to undergo -S-S- disulfide bond formation in the C-terminal region of the molecule might also influence the stability of the molecule. However, this characteristic of the high-molecular-weight neuronal LCa has so far only been observed *in vitro* (Parham et al., 1989).

V. CONCLUDING REMARKS

Studies on clathrin in a variety of eukaryotic organisms and diverse cell types have established its universality as the coat on transport vesicles in receptor-mediated endocytic and exocytic transport pathways. Coat components have been cloned and biochemically characterized, which has resulted in considerable knowledge of their interactions. A cycle for clathrin assembly and disassembly in the cytoplasm has emerged and some regulatory elements that control vesicle formation and disassembly identified. By further exploring *in vitro* reconstitution systems of clathrin-mediated processes, more regulatory factors are likely to be discovered. Additional insights into regulatory mechanisms will help to understand how regulatory factors affect the interactions between coat components and the membrane. Ultimately such insights will contribute to understanding how clathrin coats might control the extent of membrane trafficking and protein sorting. The *in vivo* importance of clathrin regulatory factors will be tested by exploiting yeast genetics to obtain deletion strains and by generating mammalian cell lines and organisms that lack expression of CCV components and regulatory factors.

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