BIOLOGICAL BASKET WEAVING: Formation and Function of Clathrin-Coated Vesicles

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Key Words adaptor, endocytosis, secretion, cytoskeleton, sorting

Abstract There has recently been considerable progress in understanding the regulation of clathrin-coated vesicle (CCV) formation and function. These advances are due to the determination of the structure of a number of CCV coat components at molecular resolution and the identification of novel regulatory proteins that control CCV formation in the cell. In addition, pathways of (a) phosphorylation, (b) receptor signaling, and (c) lipid modification that influence CCV formation, as well as the interaction between the cytoskeleton and CCV transport pathways are becoming better defined. It is evident that although clathrin coat assembly drives CCV formation, this fundamental reaction is modified by different regulatory proteins, depending on where CCVs are forming in the cell. This regulatory difference likely reflects the distinct biological roles of CCVs at the plasma membrane and trans-Golgi network, as well as the distinct properties of these membranes themselves. Tissue-specific functions of CCVs require even more-specialized regulation and defects in these pathways can now be correlated with human diseases.

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INTRODUCTION

Clathrin-coated vesicles (CCVs) mediate sorting and selective transport of membrane-bound proteins for several pathways of intracellular membrane traffic. They are responsible for receptor-mediated endocytosis (RME) at the plasma membrane (PM) and sorting of proteins at the trans-Golgi network (TGN) during the biogenesis of lysosomes and secretory granules. CCVs are the first such transport vesicles for membrane proteins to be identified, and consequently the understanding of their biochemistry and function is quite sophisticated. The dense protein coat of the CCV and its bristle-like morphology was first described by Roth & Porter (1964), who noted that these vesicles appeared to be involved in RME of yolk proteins in mosquito oocytes. Subsequently, the remarkable morphology of the clathrin coat was noted for vesicles purified from pig brain (Kaneseki & Kadota 1969).

Clathrin was identified as one of the major coat proteins of CCVs by Pearse (1975), and the clathrate or basket-like appearance of assembled clathrin was recognized in the naming of the protein (Pearse 1975). The other major protein component of CCVs, the adaptors [also called assembly proteins (APs)], were identified by Keen et al. (1979) and characterized biochemically in the early 1980s (reviewed in Pearse & Robinson 1990). Recent major advances in the characterization of CCVs include the determination of the structure of a number of CCV coat components at crystallographic resolution, the identification of novel regulatory proteins that control CCV formation in the cell, and the discovery that phosphorylation and receptor signaling influence CCV formation. In addition, the relationship of CCV
GENERAL PRINCIPLES OF COATED VESICLE FORMATION
AND THOSE SPECIFIC TO A CCV

It has become clear that CCVs are not unique in their ability to select protein cargo for transport from one membrane to another, nor are CCVs unique in their ability to mediate endocytosis from the PM. Therefore it is important to define CCV-mediated transport pathways relative to other vesicle-mediated membrane transport pathways (Figure 1). Cargo selection for anterograde transport from the endoplasmic reticulum through the Golgi apparatus during protein export is mediated by COPII-coated vesicles. Cargo selection for retrograde transport, returning resident proteins to the endoplasmic reticulum when they escape into the Golgi apparatus, is mediated by COPI-coated vesicles (Barlowe 2000). At least two endocytic pathways are independent of CCVs. Potocytosis of small molecules can be mediated by caveolae associated with cholesterol-rich plasma membrane rafts (Anderson 1998). Endocytosis of a subset of signaling receptors and toxins has been shown to be independent of both caveolae and CCVs (Lamaze et al. 2001, Skretting et al. 1999).

The biochemical principles that control CCV formation and function can be clearly defined. Some apply to the formation of other coated-vesicle transport pathways and some are unique to CCVs. Such a comparison has been made recently in two review articles (Kirchhausen 2000b, Springer et al. 1999). The self-assembling property of the clathrin coat is the key to the ability of CCVs to selectively sequester protein cargo into a membrane vesicle. Clathrin has a triskelion (three-legged pinwheel) shape, and intrinsic to the molecule is its ability to form a polyhedral lattice (reviewed in Brodsky 1988) (Figure 1). During CCV formation, clathrin lattice formation is nucleated on cellular membranes by adaptors (AP1 and AP2), which are drawn into the lattice and trigger CCV formation at the TGN and PM, respectively. In turn adaptors incorporate transmembrane molecules into the lattice by association with the cytoplasmic domains of these molecules (Figure 2). Thus in the simplest conception of CCV formation, the polymerization of clathrin provides the organizing function for protein sorting by concentrating
Figure 2  Basic steps in the nucleation, budding, scission, and uncoating of a clathrin-coated vesicle (CCV) from either the plasma membrane (PM) or the trans-Golgi network (TGN). (1) Receptors with adaptor recognition signals are present in the donor membrane along with determinants that influence adaptor localization. (2) Adaptors bind to the localization determinants with high affinity. (3) Adaptors interact (low affinity) with the cytoplasmic domains of receptors present in the membrane. Membrane-associated adaptors are in a dephosphorylated form favorable for clathrin binding. Clathrin is recruited from the cytosol, and assembly of the clathrin lattice at the membrane is triggered by adaptors. (4) The fully coated CCV detaches from the donor membrane. (5) Clathrin is uncoated, followed by uncoating of adaptor molecules. The adaptor molecules in the cytosol are phosphorylated, preventing nonproductive interaction with clathrin. The timing of adaptor phosphorylation within the uncoating process is not defined. (6) The uncoated vesicle fuses with an acceptor compartment. In the case of budding from the PM and TGN, the acceptor compartment is in the endocytic pathway. The molecules involved in the regulatory steps indicated by letters are best defined for CCV formation at the PM. In step A, receptor uptake can be constitutive or it can be stimulated by signaling. In the latter, phosphorylation, arrestin binding, and ubiquitination can all play a role. Binding of assembly protein AP2 to the PM can be mediated by lipid recognition, synaptotagmin, and stonin 2. Orientation of nucleation relative to the actin cytoskeleton could involve Hip1R. Note that mammalian clathrin is shown with random combinations of light chain (LC)α and LCβ. Step B involves AP180 or CALM, eps15, and epsin and may incorporate auxilin. Step C involves dynamin, amphiphysin, and endophilin, and actin tails may form at this stage via pacsin/syndapin. Intersectin may also be involved in this step. Step D involves synaptojanin, hsc70, and auxilin. Step E probably involves the Rab5-guanine-nucleotide dissociation inhibitor (GDI) complex, which is also implicated in step C. For CCV formation at the TGN, step A involves activation of ARF1 and, in some cases, may involve SCAMP recognition. It is not yet established whether for CCV formation at the TGN, steps B–D involve regulatory proteins that are the same or equivalent to those described for PM CCV formation.
associated proteins into a regular protein array, and adaptors provide a cargo selection function.

For the other types of coated vesicles that have been defined, the cargo selection function has been attributed to various coat subunits: \( \gamma \)-COP or ARF-GAP (ADP ribosylation factor-GTPase activating protein) for COPI, and Sec23/24p for COPII (Kirchhausen 2000b, Springer et al. 1999). There are also two versions of protein complexes related to CCV adaptors (AP3 and AP4, both present on perinuclear membrane vesicles but not at the PM) that apparently can function independently of clathrin as cargo selectors (Kirchhausen 1999). For these other types of vesicle coats, the organizing function is defined in some cases but not others. Cargo recognition by COPII coats triggers recruitment of the other COPII subunits, which then form a concentrated array of protein, constituting a coat (Springer et al. 1999) and presumably consolidating the cargo and the recognition molecules. For COPI and the clathrin-independent adaptors, what drives coat formation is not completely clear. However, it appears that for COPI, cargo selection and self-assembly are likely to be mediated by the same large protein complex. The clathrin-independent adaptors may self-assemble and select cargo, or they may be organized by proteins not yet identified. For COP coats, cargo recognition is associated with a priming event in which a small GTP-binding protein associated with the target membrane is activated. Assembly of the coat is triggered only when the activated GTP-binding protein interacts with the cargo recognition unit (Sar1p in the case of COPII and ARF1 in the case of COPI) (Kirchhausen 2000b, Springer et al. 1999). This type of priming is also a step in CCV formation at the TGN, where ARF1 plays a role, but such a priming step has not yet been defined for CCV formation at the PM, although an increasing number of regulatory proteins have been implicated in this process. Whether the components of caveole coats are recruited by priming and cargo recognition is not yet clear, although caveolin appears to self-assemble into a regular array in cholesterol-rich regions of the PM (Anderson 1998). However, cells that lack caveolin can still internalize proteins and lipids from rafts, indicating that caveolin-independent or analogous pathways exist (Garred et al. 2001).

Finally, as more details emerge regarding CCV formation, it has become evident that although clathrin coat assembly drives this process, this fundamental reaction is modified by different regulatory proteins, depending on where CCVs are forming in the cell (Figure 2). In particular, there are a large number of regulatory differences between CCV formation at the PM and at the TGN. This regulatory difference likely reflects the distinct biological roles of CCVs at the PM and TGN, as well as the distinct properties of these membranes themselves.

STRUCTURE AND BIOCHEMISTRY OF CCV COMPONENTS AND REGULATORS

The identification of new proteins involved in CCV formation (Table 1, Figure 2) and the determination of the structures of some CCV components at molecular resolution (Figure 3) have recently moved our understanding of
<table>
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<th>Proteins</th>
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<td>CHC17 (192)</td>
<td>Chclp (190); A.t., C.e., D.d., D.m., G.g.</td>
<td>AP1β, AP2β, AP3β, AP180, epsin, β-arrestin, synaptojanin (TD); CLC, auxilin, Hic70, Hip1R, ankyrin, amphiphysin</td>
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<td>CHC22 (180)</td>
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<td>Clc1p (36); A.e., D.d., D.m.</td>
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<td>Apl4p (94); A.t., D.m.</td>
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<td>Apl1p (54); C.e., D.d., D.m.</td>
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<td>(de Beer et al. 1998, Enmon et al. 2000, Salcini et al. 1999, Wendland &amp; Emr 1998, Whitehead et al. 1999)</td>
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| Abbreviations used: AGEH, adaptor gamma ear homology; Cam KII, Ca$^{2+}$/calmodulin kinase II; CHC, clathrin heavy chain; CLC, clathrin light chain; EAST, epidermal growth factor receptor-associated protein with SH3 and TAM domains; EH, Eps15 homology; ENTH, epsin N-terminal homology; IP, inositol polyphosphate; LC, light chain; LL, di-leucine motif; PDZ, PSD-95, DLG, ZO-1; PH, pleckstrin homology; PKC, protein kinase C; PLZF, promyelocytic leukemia Zn$^{2+}$ finger protein; POB1, partner of RalBP1; PRD, proline-rich domain; SH3, Src-homology 3; TD, terminal domain; VHS, Vps27, Hrs, STAM; YXX$^8$, tyrosine-based motif; W ASP, Wiskott-Aldrich syndrome protein.

b Other nonmammalian organisms in which a homolog has been identified are abbreviated as follows: A.c., Aplysia californica; A.t., Arabidopsis thaliana; C.e., Caenorhabditis elegans; D.d., Dictyostelium discoideum; D.o., Discopyge ommata; D.m., Drosophila melanogaster; G.g., Gallus gallus; L.p., Loligo pealei; O.m., Oncorhynchus mykiss; X.l., Xenopus laevis; Z.m., Zea mays. Name of the homolog protein is provided in parentheses following the species abbreviation.

c Binding partner(s) for the protein in the first column are listed and, where known, the interaction domain of the binding partner is noted. Parentheses after each partner or set of partners enclose the interaction domain of the protein in the first column that binds the partner(s) and indicate domains.

d Apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in kDa: AP180, 91; AP3$^\delta$, 160; AP4$^\epsilon$, 140; Epsin1, 94; GGA1, 85; GGA3, 30/36; Synaptotagmin I, 65.

e Neuronal splice variant(s).
CCV biochemistry to a new level (Kirchhausen 2000b, Slepnev & De Camilli 2000, Wakeham et al. 2000). In this section we review the structure and biochemistry of bona fide members of the vesicle coat, as well as those proteins involved in vesicle assembly, disassembly, and scission from the membrane, to set the stage for understanding the cellular physiology of CCV formation.

Clathrins

The clathrin triskelion is formed from three identical clathrin heavy chains (CHCs) of 1675 residues in mammalian clathrin (192 kDa) (Kirchhausen et al. 1987), with variation of sequence length at the extreme carboxyl (C) terminus in CHCs of other species (Brodsky 1999). CHCs have variable migration by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), such that mammalian CHCs appear to be 180 kDa, whereas yeast CHC, which terminates at the equivalent of mammalian residue 1653, runs closer to 190 kDa (Tan 1993). CHCs can be divided functionally and structurally into thirds (Figure 4). The central portion of the clathrin molecule, known as the hub (Liu et al. 1995), is formed by the C-terminal third (mammalian CHC residues 1074–1675) and includes sequences mediating CHC trimerization (between mammalian residues 1560–1615) and binding sites for the clathrin light chain (CLC) subunits, which regulate clathrin assembly (Brodsky et al. 1991). The proximal portion of the triskelion leg (mammalian residues 1074–1522) extending from the trimerization domain is a superhelix of $\alpha$-helices (Figure 3). These helices are grouped in a repeating structural motif comprising 10 short $\alpha$-helices linked by a pattern of conserved salt bridges (Ybe et al. 1999). The general sequence motif characteristic of the structural motif is repeated seven times in all CHC sequences, which suggests that this CHC repeat (CHCR) accounts for the entire linear portion of the triskelion leg (proximal and distal) and contributes to CHC trimerization. The CHCR also appears in other proteins (singly or in pairs) involved in membrane traffic, including Pep3p, Pep5p, Vps39p, and Vps41p. A single CHCR would be about 55 Å in length and may represent a general protein interaction domain or possibly a clathrin-binding domain (Ybe et al. 1999). The central third of the CHC forms the distal segment of the triskelion leg and is predicted to have a similar structure to the proximal leg. A flexible bend separates the proximal from the distal leg segments (Musacchio et al. 1999), and even when this connection is severed, the resulting separated fragments still self-assemble to form a lattice (Greene et al. 2000). The N-terminal third of the CHC comprises a globular terminal domain (TD), which forms a beta-propeller structure, with binding sites for various clathrin-interacting proteins on the “blades” of the propeller (ter Haar et al. 1998) (Table 1, Figure 3). The TD is connected to the distal leg segment by a linker region of short alpha helices, which are not organized into a CHCR but appear to be more flexible. The TD has been cocrystallized with peptides containing clathrin-binding sequences both from the $\beta$-chain of the AP3 adaptor and from $\beta$-arrestin, which bind to the same binding site (ter Haar et al. 2000). It is likely that this limited binding site...
represents only a fraction of an extended site for either protein to interact with clathrin.

CHC22, a second form of CHC (1640 residues), was identified during human genome analysis of human chromosome 22 (reviewed in Brodsky 1997). The conventional CHC described above is encoded on human chromosome 17 and for comparative purposes is referred to as CHC17. CHC22 is 84% identical to CHC17, and the differences are scattered throughout the protein sequence such that no particular domain is dramatically different from CHC17. CHC22 forms a trimer but does not associate with CLCs as avidly as does CHC17 (Liu et al. 2001b). CLC binding to CHC22 is not detected biochemically and has been observed only through yeast two-hybrid interactions, whereas CLCs form a stable subunit of CHC17 triskelia and are dissociated only under extreme denaturing conditions. It is possible that CHC22 has an additional subunit replacing CLCs, but no such subunit has yet been identified. CHC22 is highly expressed in skeletal muscle and can be detected at a low level in other cell types. In nonmuscle cells, CHC22 is associated with the TGN but not the PM and correspondingly associates with adaptors AP1 and AP3 but not AP2 (Liu et al. 2001b). The function of CHC22 appears to be distinct from CHC17 and is discussed further in the sections on tissue-specific function and cytoskeletal interactions below. It is of interest that a gene encoding CHC22 is not present in mice, whose corresponding chromosomal region seems to have been deleted during inversion (Lund et al. 2000). Southern blot analysis has suggested its presence in primates, rabbits, and dogs (Holmes et al. 1997), but in nonmammalian species the sequences of CHCs are too divergent to determine whether the CHC resembles human CHC22 or CHC17.

The CLC subunits bind to the hub region of the triskelion, one associated with each leg. There is a single CLC in yeast, invertebrates, and insects, and there are two forms (LCa and LCb) in vertebrate species. These are encoded on different chromosomes in humans and have about 60% protein sequence identity. There are also neuronal splicing variants of both LCa and LCb (Brodsky et al. 1991). Thus LCa and LCb vary from 25–29 kDa, but by SDS-PAGE they appear to be 30–36 kDa, as they are highly negatively charged (Brodsky 1988). CLCs bind clathrin through a central domain, flanked on the C-terminal side by the splice sites for neuronal sequences, which introduce a hydrophobic patch into the proteins. On the N-terminal side, both LCa and LCb have a calcium-binding site and a sequence unique to each CLC, which in LCa can stimulate the uncoating protein hsc70 to disassemble polymerized clathrin (Brodsky et al. 1991). Approximately 20 residues from the N terminus in both light chains is a completely conserved sequence of 22 residues shared by LCa and LCb in all mammalian species and which represents the region of highest homology with yeast CLC (Pley & Parham 1993). The first three residues of the conserved mammalian sequence are negatively charged, with two charges conserved in CLCs of all species analyzed to date. These charged residues regulate the pH sensitivity of clathrin assembly. Without CLC bound, triskelions of CHCs will polymerize into a lattice at physiological pH. With CLC bound, polymerization will only occur below pH 6.5, and this effect depends
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on the negatively charged residues at the beginning of the conserved CLC sequence (Ybe et al. 1998). Adaptors AP1 and AP2 can reverse this inhibition and stimulate assembly of triskelia at physiological pH. Thus one role of CLCs is to moderate the tendency of CHCs to self-assemble, rendering clathrin susceptible to regulation by adaptors in the cell. In vitro, clathrin self-assembly can be stimulated by calcium, and this effect is reduced when CLCs are dissociated from triskelia. It is not clear whether calcium plays a role in cellular clathrin assembly because concentrations required for CLC binding of calcium (K_d of 25 µM) are considerably higher than steady state intracellular calcium levels (Pley & Parham 1993).

In vertebrate cells, LCa and LCb compete with each other for CHC binding (Brodsky 1988). Although LCb has a slightly higher affinity in vitro, cellular triskelia comprise a random distribution of LCa and LCb according to their relative expression level in the cell, such that four types of triskelia (aaa, aab, bba, and bbb) are always present. LCa and LCb are expressed at different relative levels in cells of different tissue origins. Notably LCb is in excess of LCa in cells with a regulated secretory pathway (Acton & Brodsky 1990). However, major differences in function of LCa and LCb have not been evident from analysis of PC12 cells lacking LCa, which suggests that LCb may represent the more-specialized form and/or that the differential function of LCa and LCb is only important for cells participating in organized tissue. LCb and yeast LC are targets for phosphorylation (Brodsky et al. 1991, Chu et al. 1999), which may reflect regulation of their function.

Adaptors and Related Proteins

The heterotetrameric adaptors AP1 (γ-, β1-, μ1A-, and σ1-subunits) and AP2 (α-, β2-, μ2-, and σ2-subunits) are established components of the coat of CCVs (Kirchhausen 1999). These adaptors localize CCV formation to the TGN (AP1) or the PM (AP2) (Pearse & Robinson 1990) by induction of clathrin assembly through the β1- or β2-subunits (Gallusser & Kirchhausen 1993). They bind to cargo with the μ1- or μ2-subunits. In particular, the μ-subunits recognize sequence motifs YXXΦ (where Φ is a bulky hydrophobic residue) in the cytoplasmic domains of receptors that are sequestered in CCVs (Ohno et al. 1995). The structure of the μ2-subunit has been determined, cocrystallized with a few peptides (Owen & Evans 1998). It consists of two β-sandwich subdomains joined so that the whole structure is one convex surface with a peptide-binding site along the edge of one subdomain. The peptide (in an extended conformation) mimics an additional β-strand, and there are defined pockets that can accommodate the critical tyrosine and bulky hydrophobic residues in the internalization motif. An extended binding site was revealed when an extended peptide from p-selectin (Owen et al. 2001) was cocrystallized. The recognition of two other CCV cargo motifs by adaptors is less well defined. The dileucine-based motif (LL, LI, or ML) can be demonstrated to interact with the β1-subunit by cross-linking assays but can be demonstrated to interact with μ1- and μ2-subunits by surface plasmon resonance (Hofmann et al. 1999, Rapoport et al. 1998). Recognition of the NPXY motif directly by the TD of
clathrin has been detected by nuclear magnetic resonance spectroscopy (Kibbey et al. 1998), but there is not yet any independent experimental evidence that this occurs in vivo.

Electron microscopic analysis has revealed that the AP2 adaptors have a characteristic morphology resembling a head with ears (Heuser & Keen 1988). The head, or core domain, comprises the N-terminal two thirds of the large subunits and the μ2- and σ2-subunits and contains the determinants that localize AP1 and AP2 to the TGN and PM, respectively. As localization properties associate with the N-terminal sequences of the α- and γ-subunits, which also control which μ- and σ-subunits are bound, localization could be a function of any or all of these three subunits. The N-terminal segment of the α-subunit of AP2 in the core domain has a binding site for phosphoinositides (residues 5–80) (Gaidarov et al. 1996).

The ears, or appendages, are formed by the C-terminal domains of the α- and β2-subunits for AP2 (residues 701–938 for α and 705–937 for β). The appendage domains are attached to the core via proline-rich hinge regions. In the β-subunit, this region has a sequence motif, L (L,I) (D,E,N)(L,F)(D,E), that has been called the clathrin box (Dell’Angelica et al. 1998). Segments of the protein containing this sequence have been shown to bind to clathrin, and indeed, the hinge-appendage fragment of the β2-subunit stimulates assembly of purified clathrin and recombinant clathrin fragments (Greene et al. 2000). Clathrin box sequences have been identified in other clathrin-binding proteins, including arrestins, amphiphysins, the AP180 family, and the β-subunits of AP1 and adaptor-related complex AP3. Peptides containing this motif interact with clathrin TDs, as described above (ter Haar et al. 2000). Recent sequence analysis of the AP180 family of proteins (see below) suggests that the actual clathrin-binding motif may contain features of the clathrin box but is more extended and somewhat more degenerate (Morgan et al. 2000).

The structures of the appendage domains of both the α-subunit (Owen et al. 1999, Traub et al. 1999) and β2-subunit (Owen et al. 2000) of AP2 have been determined at crystallographic resolution. Despite their low sequence identity, these two structures are similar (Figure 3). Each appendage can be divided into two subdomains, with a distinctly different orientation relative to each other in the α- or β-subunit. The N-terminal domain is a β-sandwich (similar to an immunoglobulin domain with nine strands in the α-appendage and eight strands in the β-appendage). The C-terminal domain is a single β-sheet, flanked by one α-helix on one side and two α-helices on the other face. The C-terminal subdomain of both structures has a hydrophobic patch, centered around a critical tryptophan residue, that is the binding site for proteins with the DΦF/W motif. Each appendage binds a different subset of these proteins (Table 1, Figure 4), and their binding has different affinities, which suggests a sequence of association of these proteins with the appendage domains during CCV formation.

The γ-subunit of AP1 has an abbreviated sequence that suggests it lacks the protein interaction domain of the other appendages. This appendage binds to a protein called γ-synergin, which may supply interaction sites for other proteins (Page et al.
γ-Synergin contains an EH (Eps15 homology) domain, which suggests the potential to interact with the NPF motif found in proteins implicated in vesicle formation at the PM. In addition, γ-synergin binds to the GGA proteins (Takatsu et al. 2000), which have homology with the appendage domain of the AP1 γ-subunit and interact with ARF3 in two-hybrid assays (Boman et al. 2000, Dell’Angelica et al. 2000, Hirst et al. 2000). It is likely that binding of γ-synergin to GGAs or to AP1 is mutually exclusive. The GGAs are localized to the TGN and are involved in transport from the TGN to lysosomes in yeast (Black & Pelham 2000). They bind to the TGN via an interaction with ARF1 and have been visualized in a dense protein coat at the TGN. The GGAs interact with clathrin in vivo, and GGAs have been shown to promote recruitment of clathrin to liposomes in vitro and to TGN membranes in vivo (Puertollano et al. 2001). GGAs are monomeric but contain multiple domains that could potentially perform all the functions of the heterotetrameric adaptors including ARF binding, cargo recognition, and clathrin recruitment (Puertollano et al. 2001). Thus GGAs appear to be able to nucleate clathrin coat formation at the TGN and thereby represent a novel form of adaptor molecule. A specialized form of the AP1 adaptor having the µ1B-subunit instead of µ1A has been identified, with expression limited to polarized cells (Fölsch et al. 1999, Ohno et al. 1999). Furthermore, two additional complexes with subunit compositions similar to AP1 and AP2 have been identified and characterized (Kirchhausen 1999). These are known as AP3 (δ-, β3A or β3B-, µ3-, and σ3A or σ3B-subunits) and AP4 (ε-, β4-, µ4-, and σ4-subunits). AP3 has been implicated in specialized sorting pathways in the TGN, particularly in melanosome formation. By immunoprecipitation it has been shown to interact with CHC17 and CHC22 (Liu et al. 2001b). The former interaction is through the predicted clathrin box in the β3-hinge region (Dell’Angelica et al. 1998). However, AP3 does not copurify with CCVs (Simpson et al. 1996), and in yeast, AP3-mediated transport pathways function independently from clathrin-mediated transport pathways (Vowels & Payne 1998). Thus whether AP3 functions completely independently of clathrin in mammalian cells or perhaps less dependently than AP1 and AP2 is not clear. The µ3-subunit does interact with cargo motifs in yeast two-hybrid analysis (Dell’Angelica et al. 1997). Expression of AP4 is limited to a low level and is localized to the TGN region of cells (Dell’Angelica et al. 1999a, Hirst et al. 1999). There are no predicted interactions with clathrin for AP4. Adaptor complexes equivalent to AP1, AP2, and AP3 have been found in nonmammalian species, including yeast, which have 13 potential adaptor subunits (four µ-subunits and three of each of the others corresponding to homologues of subunits from mammalian AP1, AP2 and AP3) (Cowles et al. 1997).

Coat Proteins Influencing Assembly and Disassembly

AP180 and auxilin are two clathrin-binding proteins involved in CCV coat assembly and disassembly, which were identified as coat components. AP180 (see Table 1 for aliases) is a neuronal protein, related in structure and function to a
nonneuronal family member CALM (McMahon 1999). Versions of AP180 have been identified in *Drosophila melanogaster*, *Caenorhabditis elegans*, and squid with variable degrees of homology and inserted sequences. All the proteins of this family have at their N termini an ENTH domain, which binds phosphatidylinositol 4,5-bisphosphate (PIP2). Although the C termini are highly divergent, all retain the ability to bind clathrin. Rat AP180, bovine AP180, *Xenopus* AP180, and human AP180 are similar, whereas CALM from rats and humans are similar to each other. Both AP180 and CALM bind clathrin and are involved in endocytosis, but they coexist in some cell types (Kusner & Carlin 2000). The other members of this family identified in yeast (Yap1801, Yap1802), *C. elegans* (Unc-11), *D. melanogaster* (LAP), and squid (AP180) appear to be more closely related to CALM than to AP180, although homology in the C terminus is low (Morgan et al. 1999). For mammalian AP180, deletion and mutagenesis studies on the clathrin-binding domain implicate a DLL or SLL motif in clathrin binding (Morgan et al. 2000). This motif is present in multiple (12) copies in both AP180 and in other clathrin-binding molecules, including all the β-subunits (one to four copies) of the AP1, AP2, AP3, and AP4. AP180 also makes a complex with AP2 via DΦF/W repeats that can bind both the α- and β-subunit appendages (Hao et al. 1999, Owen et al. 2000). This complex would have multiple DLL motifs that could potentially cross-link clathrin and promote assembly. The predicted "clathrin box" motif in CALM and LAP is actually buried and inaccessible in the structure of the molecule (Ford et al. 2001, Mao et al. 2001). However, the clathrin box motif, defined in other analyses of clathrin-binding proteins, such as that of the β-subunits of AP1, AP2 and AP3, overlaps with DLL or SLL motifs so that it is not yet possible to definitively identify what actually constitutes a clathrin-binding motif.

Auxilin, originally identified as a neuron-specific component of CCVs (Ahle & Ungewickell 1990), has now been found in two forms in mammalian cells, the neuronal form and the ubiquitous form, auxilin 2. The latter form is also a cyclin G–associated kinase (GAK) that has serine/threonine kinase activity and can phosphorylate the µ-subunit of both AP1 and AP2 (Greener et al. 2000, Umeda et al. 2000). The N terminus of both auxilins has a phosphatase and tensin homology (PTEN) domain, followed by a clathrin-binding domain and a J domain at the C terminus (Greener et al. 2000, Umeda et al. 2000, Ungewickell et al. 1995). The PTEN domain could have actin-binding activity through its tensin homology, as tensin binds actin in focal adhesion plaques (Kanaoka et al. 1997). The phosphatase associated with PTEN tumor suppressor domains can dephosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP3) (Maehama & Dixon 1998). Although the phosphatase activity of auxilins remains to be determined, through a similar activity they could potentially influence the phosphoinositide binding of other CCV components. The J domain is a defining feature of the hsp40 cochaperone family and is essential for stimulating the ATPase activity of the hsc70/hsp70 chaperones, as occurs in their *Escherichia coli* homologues DnaJ and DnaK, respectively (Kelley 1998). Thus auxilins can recruit hsc70 to the clathrin coat through their ability to interact with clathrin and APs and stimulate uncoating activity. Auxilin
in *C. elegans* and yeast (Swa2p/Aux1p) has highest homology to mammalian auxilins in the J domains and little homology elsewhere in the protein (reviewed in Lemmon 2001). Mutations in auxilin-encoding genes in yeast and worms cause accumulation of uncoated CCVs and block endocytosis. Overexpression of auxilin and GAK in mammalian cells disrupts clathrin localization to membranes. These in vivo experiments support a role for auxilin as a cofactor in CCV uncoating (Umeda et al. 2000, Zhao et al. 2001). It is of interest that auxilin was initially discovered as a stimulator of clathrin assembly in vitro (Ahle & Ungewickell 1990). It may be that auxilin/GAK has assembly-promoting properties that allow its incorporation into the CCV coat, thereby priming vesicles for uncoating.

**Dynamin and Binding Partners**

Dynamin is a GTPase that oligomerizes into tetramers that can stack into open rings and form tubules (Hinshaw 2000). Its function in scission of an assembled CCV from the PM was identified through the phenotype of the *shibire* *D. melanogaster* mutant, which accumulates CCVs attached to the PM in synaptic regions (Kosaka & Ikeda 1983). Dynamin’s role in endocytosis has been confirmed by the construction of many mutants that affect endocytosis, most notably the K44A mutant, with a defect in GTP binding and hydrolysis. Overexpression of K44A-dynamin inhibits clathrin-mediated endocytosis, as well as caveolae function (McNiven et al. 2000). There is some debate as to the exact role of dynamin during CCV fission (discussed below in the section on mechanics of CCV formation). There are numerous splice variants and three genes encoding human dynamins. Dynamin 1 functions at the PM and is the most well-characterized. Dynamin 2 has been proposed to play a role in budding of CCVs at the TGN, although conflicting results discussing dynamin 2 function are reported in the literature (Altschuler et al. 1998, Kreitzer et al. 2000, McNiven et al. 2000). Dynamin 3 has restricted tissue distribution and is most strongly expressed in testis (McNiven et al. 2000). Additional roles for mammalian dynamins in membrane traffic have been suggested by expression and mutagenesis studies and include an influence on membrane-cytoskeleton interactions (Witke et al. 1998) and intracellular signaling (Ahn et al. 1999, Fish et al. 2000, Whistler & von Zastrow 1999). Dynamin homologues have been identified in *D. melanogaster, C. elegans,* and yeast. In the latter, the closest homologue, Vps1p, is involved in TGN-to-vacuole membrane traffic; a more distant homologue, Dnm1p, has been implicated in endosome-to-vacuole transport (Gammie et al. 1995) and mitochondrial morphology (Bleazard et al. 1999); and a third, Mgm1p, is also involved in mitochondrial function (Baggett & Wendland 2001).

Dynamin 1 interacts with numerous protein partners, all of which play a role in CCV-mediated endocytosis and some of which, such as profilin and cortactin, can interact directly with the actin cytoskeleton. The majority of dynamin’s protein interactions occur through a proline-rich domain at the C terminus, which binds SH3 domains in other proteins (Table 1, Figure 4) (Simpson et al. 1999). Adjacent to the proline-rich domain, dynamin has a GTPase effector domain (Muhlberg
et al. 1997) that increases dynamin’s own GTPase activity (a function of the N-terminal domain) by 50- to 100-fold. This GTPase effector domain activity can influence adjacent dynamin tetramers during dynamin self-assembly into stacked rings of tetramers. Dynamin also has a pleckstrin homology (PH) domain that interacts with PIP2 (Salim et al. 1996) to mediate membrane binding and that could also mediate binding to the β- and γ-subunits of heterotrimeric G proteins (Liu et al. 1997). The structure of the dynamin PH domain (Timm et al. 1994) resembles that of PH domains in other phosphoinositide-binding proteins (Rebecchi & Scarlata 1998), such as Bruton’s tyrosine kinase (Btk).

Amphiphysins bind dynamin through a C-terminal SH3 domain, and some forms bind clathrin and AP2, thereby linking dynamin to these two coat proteins (Slepnev et al. 2000). Amphiphysins also interact with lipid-modifying proteins endophilin, synaptotagmin, and phospholipase D1 and 2 and with proteins involved in signaling (Table 1) (Wigge & McMahon 1998). In mammals, amphiphysin I is expressed in neurons, testis, and neuroendocrine cells, whereas amphiphysin II has notable expression in skeletal muscle and brain (Butler et al. 1997, Wigge & McMahon 1998). Amphiphysin homologs in yeast Rvs167p and Rvs161p are functionally required for endocytosis, and genetic evidence suggests an interaction with components of the actin cytoskeleton (Amberg et al. 1995). In mammals, amphiphysin II plays a role in macrophage phagocytosis, which further suggests a cytoskeletal connection (Gold et al. 2000).

MECHANICS OF CCV FORMATION AND MEMBRANE INTERACTIONS

Clathrin Assembly by Adaptors and Lattice Rearrangement

The structure of the lattice produced by clathrin assembly in the presence of adaptor molecules has been determined to a resolution of 21 Å by cryoelectron microscopy and image averaging (Figure 5) (Smith et al. 1998). The features of this lattice are that every edge is formed by the interaction of two proximal leg domains and two distal leg domains, each contributed by four different triskelia. The geometry is such that underneath the trimerization domain at the vertex of each triskelion is the conjunction of three “knee bends” from adjacent triskelia and below that is the conjunction of three TDs from triskelia centered two vertices away. The structure of the TDs has been modeled within the 21 Å map, and it is clear that they extend inside the lattice to interact with adaptor molecules, which in turn would be interacting with membrane components (Musacchio et al. 1999, Smith et al. 1998).

The CLC subunits inhibit spontaneous assembly of the heavy chains at physiological pH (Liu et al. 1995). This inhibition can be overcome in vitro, at low pH. In vivo, this inhibition is overcome by adaptor interaction with clathrin. The influence of adaptors on clathrin lattice assembly has been studied in an in vitro
reconstitution system in which the entire process can be reproduced with fragments of clathrin and adaptors produced in bacteria (Greene et al. 2000). Earlier work showed that adaptors bind the TD of clathrin and that the $\beta$-chain of both AP1 and AP2 can induce clathrin assembly (Gallusser & Kirchhausen 1993). Subsequent studies revealed that an N-terminal fragment of the $\beta_2$-chain comprising the hinge and ear (appendage) domain (Figure 4) will stimulate clathrin assembly (Shih et al. 1995). From structural studies and sequence analysis, it has been suggested that this fragment has two binding sites for clathrin. A peptide containing a clathrin-binding motif from the hinge region of the $\beta_3$-subunit of AP3 was cocrystallized with the TD fragment of the CHC (ter Haar et al. 2000) (Figure 3). In addition, a D$\Phi$F/W-motif binding site identified on the ear domain is predicted to interact with a D$\Phi$F/W motif in the CHCR1 of the distal triskelion leg, near the TDs of CHCs (Owen et al. 2000). The presence of two binding sites in one $\beta$-chain suggests that adaptors could potentially cross-link two CHCs to orient them in a conformation favorable for assembly. In the cell, the interaction of the $\beta$-chains of AP1 and AP2 with clathrin is regulated by phosphorylation of serines in their hinge regions (Wilde & Brodsky 1996). When membrane bound, these regions are dephosphorylated and can interact with clathrin to stimulate assembly. In the cytosol, the $\beta$-chains are phosphorylated so that they cannot interact with clathrin and nonproductive assembly is prevented. Properties of the kinase and phosphatase involved have been recently characterized, although the specific proteins have not yet been identified (Lauritsen et al. 2000). The timing of phosphorylation and dephosphorylation within the cycle of CCV formation is not known, although the kinase is associated with fully formed CCVs (Wilde & Brodsky 1996). Thus its activation may help to initiate coat disassembly.

Studies on clathrin self-assembly have indicated that the interaction of proximal triskelion legs is important for self-assembly. Monoclonal antibodies to this region inhibit assembly, and the regulatory CLCs bind in this region (Blank & Brodsky 1987). Studies with recombinant fragments have indicated that the hub fragment, comprising only the proximal leg and the trimerization domain of the triskelion, can self-assemble, but only pseudo-lattices (poorly formed and not closed) are formed. This latter reaction is not influenced by adaptor molecules or fragments. However, when adaptor fragments are combined with a fragment of the triskelion leg containing the distal leg and TD and these are added to hubs with bound CLCs, an intact spherical lattice can be formed that reproduces clathrin coat morphology (Greene et al. 2000). This assembly reaction is influenced by adaptors and requires the presence of the distal leg, as well as the TD of CHC. These observations suggest that the adaptors influence triskelion assembly through their ability to bind both the TD and the distal domain. Given that the distal part of the triskelion leg interacts so intimately with the proximal legs in a clathrin lattice (Figure 5), these segments are in a prime position to also interact with the CLCs and thereby reverse their negative effect on assembly. CLCs do not dissociate from CHCs during clathrin assembly and disassembly, but it is not known whether they undergo any conformational change during these reactions that might alter their influence on CHC assembly.
(Pishvae et al. 1997). Studies on the disposition of the CLCs along the proximal leg of the CHCs have suggested two different potential conformations (Kirchhausen & Toyoda 1993, Nächke et al. 1992), and detailed mapping of the CLC-binding site within the proximal leg is in progress. The answer to this problem will lie in the resolution of the structures of CLCs bound to CHCs in assembled and disassembled clathrin.

At the PM, large patches of clathrin lattices composed only of hexagons are frequently observed. In order for a coat to form around membranes, 12 pentagons must be introduced (based on the mathematical requirements for sphere formation). It has been argued that because of the extensive “weaving” interactions of triskelia, rearrangement of triskelia to form pentagons from hexagons can only occur at the edge of a hexagonal lattice by using the outermost triskelia as a donor pool for a novel reassembly reaction that can introduce pentagons (Kirchhausen 2000a). However, recent analysis of the energetics of clathrin basket formation in the presence of adaptors indicates that although this is an energetically favorable reaction, the threshold energy of assembly and disassembly is such that the lattice can “breathe” and potentially lead to local rearrangement (Nossal 2001). What actually happens at the cell membrane remains unresolved; however, it seems reasonable to hypothesize that rearrangement of a clathrin lattice would require the participation of additional molecules.

The potential need for lattice rearrangement appears to be a feature of CCV formation at the PM, where apparently stable hexagonal arrays of assembled clathrin are observed (Heuser & Kirchhausen 1985). In the TGN, vesicles appear to form from tubules, and the CCVs in the TGN are less stable. Correspondingly, nucleation of CCVs in the TGN is regulated differently (see sections below). There are distinct differences between CCV formation at the PM or at the TGN with respect to both the source of clathrin (membrane bound versus cytosolic) and the nature of the donor membrane (flat and cholesterol rich versus tubulated). These differences could explain why candidate molecules for clathrin rearrangement and lipid deformation have been identified mainly in endocytic-coated vesicles, without the implication of strictly analogous participants in CCV formation at the TGN. It is likely not a coincidence that many such accessory molecules have been discovered by studying CCV formation at nerve termini, where extensive endocytosis from the PM occurs after a burst of synaptic activity (Slepnev & De Camilli 2000).

Candidates for involvement in a lattice rearrangement reaction at the PM are molecules such as Eps15 (Salcini et al. 1999) or amphiphysin I (Wigge & McMahon 1998). Both molecules interact with clathrin and adaptors. Amphiphysin binds directly to both clathrin and adaptors, as well as to dynamin. Eps15 binds the AP2 adaptor directly and clathrin via its binding partner epsin, a protein that also binds AP2. Eps15 was originally identified as a substrate for the epidermal growth factor receptor (EGFR) tyrosine kinase (Salcini et al. 1999). Through expression of dominant negative mutant fragments of Eps15, it is clear that it plays a critical role in constitutive as well as receptor-stimulated endocytosis (Benmerah et al.
Eps15 forms homodimers of parallel stalks and tetramers of antiparallel dimers (Cupers et al. 1997), or it can heterodimerize with intersectins, scaffold proteins that provide links to signaling molecules (Sengar et al. 1999). Eps15 is localized to the necks of CCVs forming at the PM and interacts avidly with the AP2 adaptor, as well as with synaptojanin, epsin, and intersectin (Salcini et al. 1999). Epsins (Slepnev & De Camilli 2000) also bind AP2, as well as clathrin, expanding the potential for multimeric interaction between CCV coat proteins during assembly.

The AP180 and CALM proteins also apparently play a PM-specific role in stimulating clathrin assembly (McMahon 1999, Tebar et al. 1999). It is not established whether either of these proteins localizes to CCVs in the TGN, although overexpression of CALM by transfection showed a TGN, as well as a PM, localization (Tebar et al. 1999). AP180 stimulates clathrin assembly and influences the size of a clathrin coat, a factor that may be particularly important at the neuronal PM, corresponding with the high neuronal expression of AP180. CCV formation at the synapse plays a major role in recapture of synaptic vesicle (SV) contents. SV can reform either from CCVs or by resorting of SV proteins from endosomes (Slepnev & De Camilli 2000). In the former situation, regulation of the size of the recapture vesicle by AP180 is critical for generation of SV of the appropriate size (Zhang et al. 1999). Studies on the function of CALM indicate that it also plays a role in mammalian cell endocytosis (Tebar et al. 1999). CALM may also regulate vesicle size, but its function may have more to do with localization of CCV formation through interaction with PM-specific lipids (see next section). Thus the two related proteins may have diverged to play slightly different functions in stimulation of CCV formation at the PM in different cell types (Kusner & Carlin 2000). AP180 is not sufficient on its own to stimulate CCV formation. It can induce clathrin lattice formation on lipid monolayers, but these lattices do not invaginate unless AP2 is also present (Ford et al. 2001).

Finally, the late stages of CCV formation at the PM seem to depend on and incorporate the Rab5-GDI complex into the CCVs (McLauchlan et al. 1997). This complex is involved in fusion of uncoated CCVs with each other and with early endosomes. It is not known whether the formation of CCVs at the TGN requires incorporation of an equivalent Rab5-GDI complex to facilitate transport to target membrane compartments.

Lipid Interactions During CCV Formation

At the PM, regions of CCV formation are not as rich in cholesterol as are caveolae and rafts, but cholesterol content is important for CCV formation at the PM and cholesterol depletion interferes with vesicle budding (Subtil et al. 1999). The energetics of clathrin assembly with adaptor molecules is favorable at physiological pH and could potentially provide enough energy to cause spontaneous vesicle budding (Nossal 2001). However, it appears that lipid deformation both at the PM and TGN during vesicle formation has molecular assistance and that lipids play a
role in nucleation of vesicle formation at both cellular sites. A number of proteins associated with PM CCVs, including the $\alpha$-subunit of AP2, dynamin, amphiphsisin I, and, most recently, AP180 and epsin, have been shown to bind phosphatidylinositol polyphosphates (PIPs) (Cremona & De Camilli 2001, Ford et al. 2001, Itoh et al. 2001). Using in vitro assays, it has been shown that interaction of dynamin and amphiphsisin (separately and in combination) can deform lipid membranes, and AP180 can cause initiation of clathrin assembly at artificial membranes (Ford et al. 2001). In the case of AP180 and AP2, lipid recognition may help to initiate clathrin assembly at the PM, and for AP2, lipid binding has been implicated in clathrin assembly on lysosomal membranes (Arneson et al. 1999). In the case of dynamin and amphiphsisin, lipid interactions may contribute to membrane deformation during budding and vesicle scission. Clathrin binds a class II PI3-kinase through its TD and thereby stimulates PIP formation, amplifying binding sites for coat proteins at both the PM and TGN (Gaidarov et al. 2001).

CCV-interacting proteins synaptojanin, amphiphsisin, and dynamin bind endophilins. Endophilin I has lysophosphatidic acid acyl transferase activity (Huttner & Schmidt 2000, Schmidt et al. 1999). This reaction generates phosphatidic acid, potentially causing a change in membrane curvature and contributing to budding and scission. Furthermore, Eps15, AP2, the TD of clathrin, intersectins, syndapin, and endophilins (Haffner et al. 2000, Micheva et al. 1997) all have binding sites for interaction with the phosphatase synaptojanin. In vitro, synaptojanin dephosphorylates PIPs at the 3, 4, and 5 positions of the inositol ring through dual phosphatase modules (Hughes et al. 2000, Woscholski & Parker 1997). Loss of synaptojanin in mice is lethal, and their neurons accumulate uncoated CCVs (Cremona et al. 1999). This suggests that PIP binding is a critical interaction stabilizing an assembled coat and that inositol-specific phospholipase may play a role in vesicle uncoating. In yeast, mutants of synaptojanin homologs are defective in endocytosis and regulation of the actin cytoskeleton (Hughes et al. 2000). A worm with defective synaptojanin (Unc-26) is impaired in vesicle recruitment, fission, and uncoating (Harris et al. 2000). These phenotypes are consistent with a pleiotropic role for synaptojanins in regulating interactions between proteins and the PM. All these interactions between lipids and CCV-associated proteins depend on PIPs, which are present primarily on the cytosolic leaflet of the PM, in the endocytic pathway, and in the TGN.

There are TGN-specific lipid interactions of coat proteins that are implicated in CCV formation at the TGN (Roth 1999). Binding of AP1 to the TGN requires the formation of ARF1-GTP and its interaction with an additional unknown factor. This activation step for coat formation is shared by other coats in the Golgi region but is not a feature of CCV formation at the PM. ARF1-GTP formation in turn depends on guanine nucleotide-exchange factors (GEFs) of two classes. The high-molecular-weight class includes yeast Sec7p, Gea1p, and Gea2p and mammalian p200 BIG1, BIG2, and GBF1. All but GBF1 are inhibited by the fungal metabolite brefeldin A (BFA). The low-molecular-weight GEFs including ARNO, cytohesin-1, cytohesin-4, and GRP1, have PH domains and are insensitive to BFA.
ARFs and both classes of GEFs bind PIPs, and their recruitment to TGN membrane is responsive to PI3-kinase activity. In addition, ARF1-GTP stimulates phospholipase D activation, potentially resulting in increased PIP synthesis and increased sites of ARF and GEF recruitment, which could serve as a positive feedback loop for coat component recruitment. Amphiphysins inhibit phospholipase D activity, although it is not established whether this contributes to their function at the PM or whether amphiphysins also function at the TGN (Lee et al. 2000).

Role for Dynamin in Vesicle Scission

In the shibire mutant of D. melanogaster, neuronal synapses are studded with CCVs attached to the PM via collared necks, which suggests a role for dynamin in vesicle scission (Sever et al. 2000). This role is supported by the observation that dynamin can also self-assemble and form coated tubules following interaction with either synthetic or coated-vesicle-derived liposomes. Furthermore, the GTPase activity of dynamin is associated with a conformational change in dynamin tubules (Marks et al. 2001). However, there has been considerable debate about the specific function of dynamin in the cell, primarily focused on the role of the GTPase activity of dynamin. Several models have been proposed based on in vitro data and the phenotype of in vivo mutants in the GTPase and GTPase effector domains (Marks et al. 2001, Sever et al. 2000). At one end of the spectrum is the hypothesis that dynamin functions as a regulatory GTPase, like most characterized GTPases, and that it attracts other proteins that actually mediate vesicle scission. In this model, the function of self-assembly of dynamin is primarily to stimulate the GTPase activity of dynamin and perhaps act as a sensor of vesicle closure. Alternative hypotheses suggest that the self-assembly of dynamin is the mechanical force behind vesicle scission, either through formation of a garrote causing membrane constriction or through intrinsic spring-like action due to a conformational change causing membrane rupture. It is likely that the role of dynamin in CCV scission involves both of these mechanisms. As more dynamin mutants are studied for their effects on endocytosis, as well as their in vitro assembly phenotype, it appears there is supportive data for more than one mechanism of action (Marks et al. 2001). Presumably dynamin self-assembly does contribute to the mechanics of scission, along with a conformational change induced by GTP hydrolysis. However, self-assembly also activates GTPase activity, which could very well play a regulatory role in the recruitment of other proteins involved in scission. In addition, dynamin has recently been implicated in late stages of vesicle invagination during CCV formation at the PM, possibly a function of its interaction with partner proteins that may occur prior to dynamin self-assembly and vesicle scission (Hill et al. 2001). Although there is conflicting data about the role of dynamin in CCV function at the TGN (Altschuler et al. 1998, Kreitzer et al. 2000), it has been suggested that the dynamin that functions at the TGN might be a different splice variant than the ones that function at the PM, again highlighting the different requirements for vesicle formation at these two membranes (McNiven et al. 2000).
Coat Disassembly

Dissociation of coat proteins from lipids and from each other are both needed for CCVs to uncoat. Dissociation of AP2 and AP180/CALM from the PM is likely to be mediated by synaptojanin (Cremona et al. 1999), whereas GTP hydrolysis by ARF may contribute to destabilization of AP1 binding to lipids at the TGN (Roth 1999). Furthermore, the phosphatase activity associated with auxilins (Lemmon 2001), involved in uncoating, could conceivably destabilize coat protein-PIP interaction. It is not clear how lipid dissociation processes are coupled to disassembly of the clathrin lattice. Clathrin and adaptors dissociate from CCVs in separate steps (Hannan et al. 1998), and their dissociation may be promoted by adaptor phosphorylation in the clathrin-binding domain of the β-subunits (Wilde & Brodsky 1996). Following clathrin dissociation, AP2 does not appear to be present in significant levels on endosomes, although it may help mediate the aggregation of CCVs that have lost clathrin (Beck & Keen 1991a). AP1 has recently been shown to bind to a kinesin superfamily protein (KIF13A), and this binding has been implicated in cargo transport in the TGN, where vesicles with AP1 but not clathrin are observed (Nakagawa et al. 2000). Thus AP1 appears to function temporally after clathrin disassembly. However, when cells are treated with BFA, causing ARF1 to dissociate from TGN membranes, AP1 and AP3 also dissociate from membranes and, in the case of AP1, associated clathrin molecules disassemble (Liu et al. 2001b, Robinson & Kreis 1992, Wong & Brodsky 1992).

Hsc70 was shown to be able to trigger clathrin basket disassembly in vitro in the 1980s (Schlossman et al. 1984). Its role in this process in vivo was not substantiated until more recently, via antibody injection studies (Honing et al. 1994) and the use of dominant-negative mutants (Newmyer & Schmid 2001) of hsc70, which disrupt cellular functions of CCVs. The recently defined role in cellular CCV disassembly for auxilin, with its DnaJ homology, also strengthens the implication of hsc70 as a regulator of clathrin disassembly (Lemmon 2001). Hsc70 is an ATP-dependent chaperone, which binds relatively hydrophobic peptides or exposed protein sequences (Bukau & Horwich 1998). The structures of the peptide-binding site and the ATPase domain have been determined independently (Flaherty et al. 1990, Zhu et al. 1996), and the latter has an ATPase domain that resembles that of actin (Flaherty et al. 1991) (Figure 3). The recruitment of hsc70 to CCVs by auxilin followed by ATP binding and hydrolysis by hsc70 may cause a conformational change in assembled clathrin, triggering disassembly. In vitro studies revealed that a sequence unique to clathrin LCa, exposed in the presence of calcium, can stimulate hsc70 ATPase activity (DeLuca-Flaherty et al. 1990). Subsequent studies indicated that hsc70, in conjunction with auxilin, can cause clathrin disassembly in the absence of CLCs (Lemmon 2001). However, the LCa sequence could still contribute a regulatory role in vivo uncoating. Indeed, cells lacking LCa have a reduced rate of CCV uncoating (Acton et al. 1993).

Following ATP hydrolysis and coat dissociation, ADP-bound hsc70 and clathrin form a stable complex, presumably maintaining the pool of cytosolic clathrin
in a disassembled state. Included in this complex is valosin-containing protein (100 kDa), an ATP-binding protein with a suggested chaperone function (Pleasure et al. 1993). A second protein, which may also contribute to sequestration of cytosolic clathrin, is the giant protein (p619) with numerous regulatory protein domains (Rosa & Barbacid 1997). These include a domain homologous to cell cycle regulator RCC1, a guanine nucleotide exchange factor for Ran, seven β-repeats characteristic of the β-subunit of heterotrimeric G proteins, three SH3 domains, a leucine zipper, and a domain homologous to the E3 ubiquitin-protein ligases. The giant protein forms a cytosolic ternary complex with clathrin and hsp70, but it also stimulates guanine nucleotide exchange on ARF1 and rab proteins, perhaps contributing to clathrin recruitment at the TGN.

INTRACELLULAR LOCALIZATION OF CCV FORMATION AND CARGO INTERACTION

In mammalian cells, intracellular localization of CCV formation is specified by the localization of the adaptor molecules AP1 and AP2. Binding of both adaptors to cellular membranes is independent of clathrin function. Studies of adaptor interactions with membranes suggest that both protein determinants (Mahaffey et al. 1990, Mallet & Brodsky 1996, Seaman et al. 1996) and lipid determinants are recognized (see previous section on lipid interactions). In yeast, CCVs can form and function in the complete absence of adaptors or yeast AP180 (Huang et al. 1999, Yeung et al. 1999). Thus nucleation of clathrin assembly on yeast membranes can occur through other mediators of interaction between clathrin and lipids. Presumably the yeast adaptors share some of the properties of mammalian adaptors, and their incorporation into yeast CCVs might at least have a role in cargo recognition, even if adaptors are not absolutely required for CCV formation. It is notable that key cargo recognition signals in yeast differ from those that play a key role in mammalian receptor sequestration by AP1 and AP2 into CCVs, which suggests that other types of “adaptor” molecules may also be involved in receptor recognition in yeast. As with the mechanics of clathrin assembly and disassembly discussed above, the process of CCV nucleation and cargo recognition is different for CCVs at the PM or TGN in both yeast and mammalian cells. For this reason, the events at each membrane are discussed separately.

Plasma Membrane CCV Nucleation and Receptor Sequestration

AP2 binding to PM fragments in vitro can be ablated by proteolysis, which suggests that AP2 recognizes protein determinants in addition to PIPs (Mahaffey et al. 1990). Candidate determinants for nucleation of AP2-PM binding include the synaptotagmins (von Poser et al. 2000). Neuronal and nonneuronal synaptotagmins bind AP2 through their C2B domains. Synaptotagmins associate with the PM via calcium-dependent phospholipid binding and by recognition of PIPs. Expression of a
nonneuronal synaptotagmin lacking the AP2-binding domain can inhibit endocytosis in nonneuronal cells as a dominant-negative mutant. In addition, cells expressing this mutant were observed to have fewer coated pits. However, synaptotagmin is expressed at high levels in neuronal cells and the nonneuronal synaptotagmins are expressed at considerably lower levels. Thus it is not clear whether these proteins play a general role in nucleating AP2 binding.

Two additional protein families have been identified that may play a role in nucleation or localization of CCVs to the PM. Both proteins interact with Eps15, and overexpression of functional fragments affects endocytosis. These are the Numb proteins and stonin 2, both ubiquitously expressed mammalian homologues of D. melanogaster genes involved in neuronal function. The Numb family of proteins localize to CCVs at the PM and in the TGN and bind the \( \alpha \)-appendage of AP2 (Santolini et al. 2000). Stonin 2 has some homology to the \( \mu \)-subunits of adaptors and interacts both with Eps15 and with synaptotagmin, which suggests it could serve as a link between PM-associated proteins and CCV components (Martina et al. 2001).

CCVs mediate endocytosis of nonsignaling receptors, such as receptors for nutrients, whether or not ligand is bound by these receptors. The role of such cargo in coated pit nucleation is not completely established. Peptides containing the YXX\( \Phi \) endocytosis motif present in a number of these receptors can enhance the interaction of AP2 with synaptotagmin in vitro and can enhance the binding of AP2 to membrane fragments of nonneuronal cells (Haucke & De Camilli 1999). However, at very high concentrations, soluble cytoplasmic domains of receptors can inhibit AP2 binding to membranes (Chang et al. 1993). Conversely, interaction of AP2 with PIP3 can increase the affinity of AP2 for cytoplasmic tail peptides (Gaidarov & Keen 1999). Overexpression of transferrin receptor in chicken cells has been reported to increase coated pit numbers (Miller et al. 1991). However, in other cell types, no effect of receptor overexpression on coated pit formation has been observed (Brown et al. 1999, Warren et al. 1997). It has recently been noted that phosphorylation of the \( \mu \)-subunit of AP2 increases its affinity for peptides with tyrosine-based motifs and can increase AP2 binding to membrane fragments (Fingerhut et al. 2001). The physiological pathways that influence \( \mu \)-subunit phosphorylation have not been characterized. A reasonable conclusion based on the sum of these observations about cargo-AP2 interactions is that AP2 membrane interactions partially involve recognition of receptor cytoplasmic domains, but that these latter interactions are secondary to independent interactions mediating AP2-membrane binding, although the two binding events may be mutually stimulatory.

Receptors that trigger signaling pathways on ligand binding are generally endocytosed in a ligand-dependent fashion. In mammalian cells, these fall into two well-characterized categories—receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCR). In yeast, several cell surface proteins are internalized upon ubiquitination. Ste6p (a peptide transporter) and pheromone receptors Ste2p and Ste3p are monoubiquitinated in response to phosphorylation induced by ligand binding. This monoubiquitination is the cargo recognition signal for receptor
down-regulation by CCVs (Wendland et al. 1998), but whether this ubiquitin-mediated uptake depends on clathrin is not clear. In mammalian systems, ubiquitination has also been associated with the uptake of growth hormone receptor (van Kerkhof et al. 2001). Thus ubiquitination may represent one of several general mechanisms by which receptors are sequestered in transport carriers, possibly CCV, in response to ligand binding. The adaptor molecule that recognizes the ubiquitin signal in yeast has not been identified, although mutation studies in mammalian cells suggest that ubiquitin may have sequences related to the dileucine motif recognized by AP2 and AP1 adaptors (Nakatsu et al. 2000). Uptake of yeast a-factor receptor Ste3p could also rely on the presence of the sequence NPFXD in the cytoplasmic domain, which is known to be recognized by CCV components. How recognition of this sequence is ligand gated has yet to be established (Tan et al. 1996).

For mammalian signaling receptors, it does not appear that ligand binding stimulates CCV formation (Santini et al. 1998). However, ligand binding does stimulate recognition of the receptor by AP2 molecules. In the case of RTKs, phosphorylation of the receptor cytoplasmic domain upon ligand binding promotes a conformational change that exposes a binding site for AP2 (Chen et al. 1989). For epidermal growth factor receptor (EGFR), this binding site has particularly high affinity, unlike the AP2-binding site in constitutively internalized receptors, and receptor-AP2 interaction is detectable by immunoprecipitation following receptor activation (Sorkin & Carpenter 1993). Coprecipitation of constitutively internalized receptors with AP2 is not easily detected. The high-affinity AP2-binding site can be eliminated from EGFR and the receptor can still be endocytosed by CCVs, which suggests that additional interactions with the endocytic machinery function in EGFR uptake. Consistent with this result is the observation that mutations in the υ-subunit of AP2 that block recognition of tyrosine-containing internalization motifs can abrogate transferrin receptor internalization but not affect EGFR uptake (Nesterov et al. 1999). Taken together, these data on the RTK EGFR suggest that the endocytosis of signaling receptors in response to ligand binding is controlled by several independent interactions, in addition to υ-subunit recognition of the receptor’s cytoplasmic domain. Ligand binding to both EGFR and nerve growth factor receptor results in stimulation of src family kinases, which phosphorylate CHC on residue 1477, in the hub domain (Beattie et al. 2000, Wilde et al. 1999). After ligand binding to either RTK, clathrin recruitment to the PM is observed, and for EGFR, this recruitment can be abrogated by inhibition of clathrin phosphorylation (Wilde et al. 1999). The implication of these observations is that ligand binding leads to clathrin phosphorylation, which enhances clathrin localization to the PM for participation in ligand-gated endocytosis.

Enhanced interaction with CCV components is also observed during internalization of ligand-activated GPCR. In this case, ligand binding leads to phosphorylation of the GPCR, which is then recognized by cytoplasmic proteins of the β-arrestin family (Ferguson 2001). The β-arrestin members of the arrestin family interact directly with the TD of clathrin (Goodman et al. 1997) and are involved
in promoting endocytosis of a subset of GPCRs. The \( \beta \)-arrestin sequence contains a predicted clathrin box (Krupnick et al. 1997), and a peptide representing this sequence has been cocry stallized with CHC TDs (ter Haar et al. 2000). The fact that its binding site overlaps with that of the clathrin box peptide from the \( \beta_3 \)-subunit of AP3 initially suggested that arrestins might be substitute adaptors for GPCR cargo in CCVs. However, it appears that arrestins also bind AP2, independently of clathrin, and that AP2 binding is critical for their role in GPCR uptake. Thus arrestins are linker proteins for GPCR sequestration in clathrin-coated pits with AP2 at the PM. In conjunction with a role at the PM, \( \beta \)-arrestin has been shown to have a PIP-binding site, which must be intact for arrestin function in GPCR uptake (Gaidarov et al. 1999a).

Following ligand binding, many signaling receptors are initially localized in lipid rafts at the PM, where kinases that mediate downstream signaling are also localized (Dykstra et al. 2001). Some of these receptors are internalized by clathrin-independent mechanisms following signaling, whereas others are eventually down-regulated in CCVs (Mineo et al. 1999). The mechanisms operating for internalization pathways that are not mediated by clathrin have yet to be established, as does the relationship between receptor signaling from rafts and eventual uptake by CCVs.

**TGN CCV Nucleation and Receptor Sorting**

AP1 membrane-binding dynamics are different from those of AP2 in a number of features. ARF1 activation by GTP binding is required for AP1 to bind membranes at the TGN (see previous section on lipid interactions). How this signal is physiologically linked to the requirement to sort proteins is not determined. It has also been proposed, based on indirect evidence, that there is an AP1 docking protein whose interaction with AP1 depends on ARF activation (Traub et al. 1993). Several membrane-associated, AP1-binding proteins have been identified, but none has been demonstrated to have a docking function (Mallet & Brodsky 1996, Seaman et al. 1996).

\( \gamma \)-Synergin binds to the ear domain of the \( \gamma \)-subunit of AP1 and has the potential to mediate interaction of AP1 with NPF-containing proteins through its EH domain (Page et al. 1999). One such family of proteins that can bind \( \gamma \)-synergin is the SCAMP family (Fernandez-Chacon et al. 2000). These proteins were originally discovered as components of exocrine secretory vesicles, and some members of the family have a ubiquitous tissue expression pattern. There is evidence that the SCAMPs cycle between the PM, the TGN, and endosomes. The distribution of SCAMPs correlates with a potential role in AP1-dependent CCV nucleation. Perhaps, similar to synaptotagmin, their role is critical in such specialized cell function as CCV nucleation during secretory granule formation, but their involvement in general CCV function is not yet defined. However, expression of a dominant-negative mutant fragment of SCAMP1 can inhibit transferrin endocytosis, indicating that SCAMP interacts with proteins critical for the transferrin uptake pathway...
and can disrupt its cycling (Fernandez-Chacon et al. 2000). γ-Synergin itself does not act as a nucleating protein for CCV formation at the TGN because its highest affinity interaction in the cell appears to be its binding to the γ-appendage of AP1 (Page et al. 1999). However, it is still a candidate for involvement in nucleation by interaction with other proteins. The recent demonstration that GGA proteins, which bind γ-synergin, can recruit clathrin to TGN membranes in an ARF-dependent fashion suggests these proteins play a role in nucleation of CCV formation at the TGN (Puertollano et al. 2001). Binding of GGA to ARF can displace AP1. It is possible that GGAs and AP1 participate in formation of different CCVs or act sequentially during CCV nucleation.

Cargo recognition by CCVs in the TGN can be direct, by μ1-subunit interactions with cytoplasmic domains bearing the YXXΦ motif (Marks et al. 1997). In addition, the AP1-binding protein PACS1 acts as a connector, recognizing phosphorylated cytoplasmic domain sequences containing an acidic cluster of amino acids with two serines that are a target for casein kinase II phosphorylation (...EECPSPSEEED...) (Wan et al. 1998). PACS1 is involved in CCV-mediated TGN sequestration of furin and sorting of both forms of mannose-6-phosphate receptor (M6PR) through recognition of their phosphorylated cytoplasmic domains, and its function has been compared with that of β-arrestin during uptake of GPCR at the PM. It is notable that both cation-independent and cation-dependent M6PRs interact with other tail-binding proteins, including TIP47, which may be involved in their recycling to the TGN (a clathrin-independent step) and with AP2, which recognizes a site that overlaps with TIP47 binding (Orsel et al. 2000). Overexpression of M6PR has been observed to increase AP1 localization to the TGN (Le Borgne et al. 1993), which may be a function of PACS1 binding. In yeast, CCVs play a major intracellular role in maintaining sequestration of proteins in the TGN, generally measured by the TGN targeting of Kex2p, a protease similar to furin (Payne & Schekman 1989). CLC is a target for extensive phosphorylation in yeast, and expression of mutant CLCs that cannot be phosphorylated partially disrupts Kex2p localization (Chu et al. 1999). Thus in yeast, CLC phosphorylation may play a role in regulation of TGN CCV function, whereas in mammalian cells, CHC phosphorylation regulates CCV function at the PM.

CCV-Cargo Interactions at Other Cellular Membranes

Clathrin coats have been observed on both endosomes and lysosomes (Stoorvogel et al. 1996, Traub et al. 1996). Endosomal clathrin coats appear to contain either AP1 or neither AP1 nor AP2. The lysosomal coats are associated with AP2. It had been proposed that the endosomal CCVs might be sorting proteins for recycling. However, there is no kinetic argument that receptor recycling is facilitated by CCVs, as the rates of transferrin receptor recycling correspond to rates of bulk flow lipid recycling (Mayor et al. 1993). In addition, transferrin receptors lacking their cytoplasmic domains recycle at the same rates as wild-type receptors (McGraw & Maxfield 1990). Consistent with these observations is the finding that expression
of the clathrin hub in cells, which has a dominant-negative mutant effect on CCV-mediated pathways, has a minimal effect on transferrin receptor recycling rates (Bennett et al. 2001). However, in HeLa cells expressing the hub molecule, the intracellular distribution of endosomes is altered. This suggests that clathrin coats on endosomes serve a transport function that influences organelle localization, but that they do not function in direct sorting of receptors from endosomes to the PM. Recycling to the basolateral membrane in polarized cells appears to be affected by BFA, which suggests that CCVs might play a role in influencing the directionality of transport from endosomes (Futter et al. 1998). The function of clathrin coats observed on lysosomes remains to be determined. The fact that these coats can form under physiological conditions in semi-intact cells suggests that such CCVs might mediate some kind of retrograde transport from lysosomes (Traub et al. 1996).

RELATIONSHIP OF CCVS TO THE CYTOSKELETON

Mammalian PM has cortical actin, but yeast has a more elaborate actin cytoskeleton at the PM. Correspondingly, endocytosis in yeast is highly dependent on the actin cytoskeleton, and CCVs appear to play a less essential role (Baggett & Wendland 2001, Wendland et al. 1998). Yeast lacking CHCs or CLCs exhibit reduced, but not completely impaired, endocytosis (Baggett & Wendland 2001). In contrast, mutations affecting the actin cytoskeleton generally abrogate endocytosis in yeast, so it has not been possible to clearly establish the relationship between CCV-mediated uptake and actin-dependent uptake. Analysis of *Dictyostelium discoideum* expressing CHCs labeled with green fluorescent protein at the C terminus reveals that a subset of labeled CCVs moves along linear intracellular tracks, which again suggests a cytoskeletal interaction for CCV proteins (Damer & O’Halloran 2000).

In mammalian cells, CCV components interact with actin-binding proteins, but the interplay between CCV formation and the actin cytoskeleton also has yet to be clearly defined (Qualmann et al. 2000). Inhibitors of actin function have variable effects on endocytosis, depending on the cell type treated (Fujimoto et al. 2000). However, endocytic CCVs in HeLa and other cell types are observed to align with actin filaments in the cell periphery, and actin depolymerization disrupts this distribution pattern (E.M. Bennett & F.M. Brodsky, unpublished data). Furthermore, a study of the dynamics of clathrin labeled with green fluorescent protein shows that recruitment of clathrin to fixed sites at the PM is dependent on an intact actin cytoskeleton (Gaidarov et al. 1999b). These interactions of CCVs with the actin cytoskeleton could be explained by a number of molecular links between CCV components and cytoskeletal components. Mammalian clathrin binds ankyrin (Michaely et al. 1999), which has a well-established function in red blood cells, linking the actin-spectrin network to the plasma membrane. Clathrin also binds to the Hip1R (huntingtin-interacting protein 1–related) protein, whose yeast
homologue, Sla2p, functions in actin-dependent endocytosis in yeast (Engqvist-Goldstein et al. 1999, 2000). Hip1R also has an ENTH domain, which could mediate its binding to PIPs, and it has been localized to CCVs at the PM and TGN. These molecular interactions predict a role for actin in defining sites of CCV formation by delivery of coat components to nucleation sites and/or by establishing nucleation sites relative to the cortical actin network so that vesicle budding can occur in between actin filaments (Fujimoto et al. 2000). Two more roles for actin in CCV function are also suggested by molecular data. First, actin could potentially play a role in vesicle scission, via the interaction of amphiphysin and dynamin (Wigge & McMahon 1998). The yeast homologs of amphiphysin are both actin-interacting proteins and their deletion affects endocytosis (Munn et al. 1995). A third potential role for actin in CCV function could be to propel CCVs away from the membrane by formation of actin tails. CCVs are short-lived, particularly at the PM; however, an actin tail could play a transient role in detachment or translocation of vesicles. In support of this hypothesis, the syndapin/pacsin proteins that interact with dynamin and synaptotagmin also interact with Wiskott-Aldrich syndrome protein family members (Qualmann & Kelly 2000). The Wiskott-Aldrich syndrome proteins can nucleate actin tail formation (Modregger et al. 2000, Qualmann et al. 1999).

The structure of the TGN is dependent on the microtubule cytoskeleton. The fact that AP1 and a kinesin interact suggests that coated or uncoated vesicles could move along microtubules at the TGN. However, CCVs with actin tails have been observed in the vicinity of the TGN (Frischknecht et al. 1999), indicating that actin may also play a role in CCV excision or transport at the TGN. Finally, the novel form of clathrin highly expressed in muscle, CHC22, appears to be a form of clathrin that has evolved stronger interactions with the actin cytoskeleton (Liu et al. 2001b) than those exhibited by conventional clathrin CHC17. In nonmuscle cells, the intracellular distribution of CHC22 at the TGN is dramatically altered by disruption of the actin cytoskeleton, whereas CHC17 distribution is relatively stable. When the function of CHC22 in muscle tissue is established, it is likely to reveal more about the relationship between the clathrins and actin.

TISSUE-SPECIFIC CCV FUNCTION AND SPECIALIZED CLATHRIN FUNCTION

Cell Polarity

In addition to fundamental roles in receptor-mediated endocytosis and in sorting proteins destined for the endocytic pathway (such as lysosomal proteases) from the TGN, CCVs play a number of specialized roles in different tissues. Most tissue culture cell lines are not polarized, as the same cells might be in situ. When polarization is induced in epithelial cell lines, it is clear that CCVs play important roles in maintaining polarity. CCVs have been implicated in basolateral targeting of
some receptors. Basolateral targeting signals for these receptors are similar to and
frequently overlap with recognition signals for binding of the \( \mu \)-subunits of AP1
and AP2 (Mostov et al. 2000). As mentioned in the section on adaptor biochemistry,
there is a \( \mu 1B \) alternate subunit of AP1, creating an AP1 that functions specifically
in the basolateral sorting pathway for the low-density lipoprotein and transferrin
receptors (Fölsch et al. 1999). Defects in \( \mu 1B \) do not affect apical targeting of
proteins. The apical membrane of polarized cells has more raft-like properties
than does the basolateral membrane, but experiments with the clathrin hub mutant
indicate that at least some of the endocytosis that occurs from the apical membrane
of cells is clathrin mediated (Altschuler et al. 1999).

**Neuronal Function**

In neurons, CCVs recapture synaptic vesicle (SV) proteins, and CCV formation
at the neuronal synapse has been well-characterized. In fact, many of the pro-
teins involved in regulation of CCV formation were identified through analysis
of molecular components involved in neuronal CCV formation, and their func-
tions have been reviewed recently (Slepnev & De Camilli 2000). In keeping with
specialized features of CCV formation in neurons, many CCV components and
regulators have neuron-specific forms. Both forms of mammalian CLCs have neu-
ronal splice variants, as do the \( \alpha \)- and \( \beta 2 \)-subunits of AP2 (Pley & Parham 1993).
The additional sequences in the neuronal forms of these proteins may mediate
their transport to the synapse or may even influence their regulation. It is no-
table that only CCVs with neuronal CLCs interact with calmodulin (Pley et al.
1995). For many proteins involved in regulation of CCV formation, their neuronal
forms are encoded by different genes, with some modified by variable splicing.
These include AP180, auxilin, amphiphysin, endophilin, intersectin, synaptojanin,
syndapin, pacsin, SCAMPs, and synaptotagmin (see Table 1 for references). As
discussed above, in the section on the mechanics of CCV formation, one of the
requirements for CCV at the synapse is control of vesicle size (Slepnev & De
Camilli 2000). An additional requirement that distinguishes neurons from other
cells is a need for synchronicity in vesicle formation and transport. The existence
of specialized neuronal forms of CCV proteins likely reflects these specialized
needs, although comparative studies between neuronal and nonneuronal forms of
these proteins have yet to be done. Although it is not clear whether AP3 adap-
tors participate in CCV formation, they have been implicated in SV formation.
The \( \beta 3 \)-subunit has two forms, one of which is primarily expressed in neurons
(Newman et al. 1995). In keeping with the importance of clathrin at the neu-
ronal synapse, many CCV components have been identified in *C. elegans* and
*D. melanogaster* by isolation of mutants in neuronal function. Furthermore, CLC
is one of the proteins that is up-regulated in the sensory neurons of aplysia dur-
ding the development of long-term facilitation (Hu et al. 1993), and CHC is up-
regulated following stimulation of recognition memory in rats (Solomonia et al.
1997).
Regulated Secretion

CCVs play an important role in cells that undergo regulated secretion. Partial clathrin coats are observed on immature secretory granules (ISGs), which also have binding sites for ARF1 and AP1 (Austin et al. 2000). ISGs form from the TGN and contain aggregated protein or proprotein destined for the mature secretory granule (SG). It has been proposed that the clathrin coats on ISGs represent CCVs that are budding to remove TGN membrane proteins that should be sorted to destinations other than the SG. A number of morphological studies and studies of the relative biosynthetic transport pathways of lysosomal enzymes and SG proteins support this hypothesis (Ditié et al. 1996, Kuliawat et al. 1997). Introduction of the clathrin hub mutant into insulin-producing cells results in enhanced proteolysis of insulin fragments (Molinete et al. 2001). This observation suggests that the role of a CCV is to remove lysosomal enzymes from an ISG as it matures into an SG and that this removal is inhibited in hub-expressing cells. The formation of melanosomes appears to involve transport pathways that are distinct from those contributing to SG formation in that they depend on AP3 function (see human diseases and mouse mutants section on CCV malfunction).

Immune System Function

In the immune system, CCVs are involved in regulation of the cell surface expression of antigen receptors and signaling molecules and in sorting molecules to the pathway of antigen processing and presentation by class II histocompatibility molecules. CCVs have been implicated in the uptake of both the T cell receptor (TCR) (Boyer et al. 1991) and the B cell receptor (BCR) (surface immunoglobulin) (Salisbury et al. 1980) following cross-linking by receptor-specific antibodies. For T cells, endocytosis and recycling of the TCR during contact with antigen is believed to contribute to continuous signaling by a T cell and sustaining T cell activation (Liu et al. 2000). For B cells, uptake of the BCR can deliver antigen to the antigen processing pathway, leading to antigen display on the cell surface and stimulation of helper T cells needed for B cell differentiation. Recent studies have suggested that the membrane region where TCRs are concentrated during T cell stimulation by a target cell (the so-called immunological synapse) has raft-like properties (Langlet et al. 2000). Signaling B cell receptors have also been localized to rafts (Dykstra et al. 2001). Therefore, the mechanism of internalization of stimulated TCR and BCR should be further investigated as mechanisms of uptake from rafts are better defined. CCVs also control the surface expression of CTLA-4, a co-receptor on T cells that negatively modulates T cell activation and contributes to the development of T cell tolerance. During T cell activation, CTLA-4 is phosphorylated on a tyrosine residue that forms part of an AP2 recognition site (Nakaseko et al. 1999). The phosphorylation therefore blocks uptake of the CTLA-4 molecule, allowing it to perform its signaling function and modulate T cell activation. On loss of TCR signaling, CTLA-4 is no longer phosphorylated.
and can be endocytosed, leading to its down-regulation and resting state localization in the TGN. CCVs play a major role in the intracellular transport of class II major histocompatibility complex (MHC) molecules, whose recognition by helper T cells leads to stimulation of an antibody-mediated immune response. Class II molecules must intersect the endocytic pathway during their biosynthetic assembly, so that they can acquire an antigenic peptide. The intracellular compartment in which class II molecules are loaded with peptides has lysosome-like properties, and similar to lysosomes, CCVs are involved in targeting molecules to this organelle (Geuze 1998). The invariant chain molecule that chaperones class II molecules through their unique biosynthetic pathway is sorted in CCVs (Hofmann et al. 1999, Liu et al. 1998). In addition, the HLA-DM molecule that catalyzes the interaction between class II molecules and peptides is localized to the peptide loading compartment by transport in CCVs (Liu et al. 1998). The specialized class I MHC molecule analog CD1 is also targeted to the peptide loading compartment through a signal for uptake in CCVs (Sugita et al. 2000). This intracellular localization facilitates the binding of glycolipid antigen by CD1, a pathway involved in the immune response to mycobacteria.

**Muscle Function**

The human CHC encoded on chromosome 22, CHC22 is most highly expressed in skeletal muscle (Liu et al. 2001b). CHC22 is also expressed at low levels in nonmuscle cells, where it is associated with the TGN via interaction with AP1 and AP3, as well as with elements of the actin cytoskeleton. Expression of the hub fragment of CHC22 in nonmuscle cells disrupts protein sorting in the TGN, but whether this is due to a direct effect on protein sorting or whether CHC22 is involved in some structural aspect of TGN organization is not known. CHC17 is also expressed in differentiated skeletal muscle, which suggests the hypothesis that CHC22 performs a muscle-specific function. There is also a form of amphiphysin II that is highly expressed in muscle cells (Butler et al. 1997, Wigge & McMahon 1998) that is a potential interaction partner for CHC22. However, this form lacks known clathrin binding sequences. Specialized pathways of membrane transport in muscle cells include the biogenesis of the T-tubule system. A muscle-specific form of caveolin has already been implicated in this process (Parton et al. 1997), and amphiphysin II is localized to T-tubules (Butler et al. 1997), so it would not be surprising if specialized clathrin might also play a role. In addition, the flux of glucose transporters is highly controlled in muscle cells, and a specialized form of clathrin could be involved in their sequestration in the endosomal pathway or their regulated expression on the cell surface (Simpson et al. 2001). It is also possible that muscle-specific forms of CCV proteins play a role in establishing protein configurations at post-synaptic sites in muscle. Alternatively, CHC22 might not function primarily in intracellular membrane transport pathways in muscle but could potentially play a role in the structural organization of muscle proteins.
CCV MALFUNCTION: INHIBITORS, MUTANTS, AND DISEASES

Molecular Inhibitors

For many years molecular inhibition of CCV function was limited to the use of low-pH shock treatment, potassium depletion, and treatment of cells with BFA (Robinson & Kreis 1992, Wong & Brodsky 1992). None of the treatments is ideal for implicating CCV in transport, as all have pleiotropic effects on cell function. Low pH and potassium depletion do appear to cause nonproductive assembly of clathrin cages in the cytoplasm adjacent to coated pits (Heuser 1989, Heuser & Anderson 1989). However, BFA affects the binding of AP3, COPI, and COPII to membranes in the Golgi and TGN, as well as disrupting the binding of AP1 (Roth 1999). A more recent approach in a substantial number of the studies in which the function of CCV-associated proteins in mammalian cells has been observed utilizes the overexpression of fragments or mutants of these proteins to test for dominant-negative effects. A potential problem with such approaches is the continued existence of an endogenous pool of normal protein and the possibility that a protein fragment binding a regulatory protein may be inhibitory by an indirect rather than a direct effect. Because so many of the regulatory proteins interact with a network of other proteins (Figure 4), the hierarchy of interaction and the direct effects of a mutant fragment are difficult to define. For example, expression of the SH3 domains of proteins that regulate CCV formation generally affects endocytosis, but because these domains can interact with so many partners, the direct and indirect effects are difficult to distinguish. Several molecular inhibitors have been widely used or are particularly well-characterized, so their inhibitory behavior can be more easily interpreted. Dynamin I, with the same mutation as the shibire D. melanogaster mutant dynamin, has been widely used. Initially, it was characterized as an inhibitor of CCV-mediated endocytosis (Damke et al. 1994), and later it was shown to also inhibit internalization from caveolae (McNiven et al. 2000). It is reasonable to assume that if a transport step is not affected by this dominant-negative mutant, then it involves neither of these pathways. One caution regarding this mutant is that cells expressing it over time tend to compensate by up-regulating non-CCV-mediated uptake pathways (Damke et al. 1994). The hub fragment of clathrin has been used in a number of studies to block CCV-mediated uptake and CCV sorting at the TGN (Altschuler et al. 1999, Liu et al. 1998, Lu et al. 1998, Trejo et al. 2000). This inhibition appears to be a direct effect of CCV function, as the mechanism of action involves hub fragments binding up endogenous CLCs so that CHC assembly and disassembly can no longer be regulated. Expression of the µ2-subunit of AP2 with a mutation in the site recognizing tyrosine-containing internalization motifs results in very specific inhibition of AP2-mediated endocytosis of constitutively internalized receptors (Nesterov et al. 1999). As noted above, this mutation does not affect the uptake of EGFR, which appears to have a specialized interaction with endocytic CCV components,
independent of the tyrosine-containing motifs present in the cytoplasmic domain of the receptor.

**Deletion Mutants in Model Organisms**

Genes encoding proteins involved in CCV formation have been identified and deleted in *Saccharomyces cerevisiae*, *D. melanogaster*, *D. discoideum*, *C. elegans*, and *Mus musculus*. Of interest is the fact that deletions of many of the regulatory proteins are not lethal. However, given the extensive redundant interactions of CCV proteins with proteins linked to the cytoskeleton (Figure 4), lack of lethality is perhaps not so surprising. It was initially a shock when the first yeast strain deleted for CHC was found to survive (Payne & Schekman 1985). However, now that we know that membrane traffic in yeast is highly dependent on the actin cytoskeleton, it appears that clathrin is used primarily to fine-tune transport, and yeast membrane traffic can occur to a limited extent, sufficient to sustain life, without clathrin (Baggett & Wendland 2001). In other strains of yeast, clathrin deletion has been found to be lethal, and the identification of suppressor mutations has been useful for identifying additional players in membrane traffic. As mentioned above, deletion mutants lacking elements of CCV formation in *D. melanogaster* and *C. elegans* often present with a neuronal phenotype. Some of the nonneuronal phenotypes are noted here. *D. discoideum* without clathrin has defects in many processes that are also associated with the actin and microtubule cytoskeleton. These include cytokinesis, establishment of cell polarity, pseudopod formation, and uropod stability and motility (O’Halloran 2000, Wessels et al. 2000). The cytokinesis defect could be due to a need for clathrin to transport membrane proteins to sites where cytokinesis is initiated (O’Halloran 2000). Clathrin has been observed in mammalian mitotic spindles (Okamoto et al. 2000), and there is a strong connection between membrane traffic and cytokinesis (O’Halloran 2000). *D. melanogaster* without clathrin has defective spermatogenesis (Fabrizio et al. 1998), likely owing to the importance of membrane compartmentalization during this process. Organisms with defects in AP3 proteins (*D. melanogaster* and *M. musculus*) have pigmentation defects (Odorizzi et al. 1998), whereas defects in AP1 subunits in *M. musculus* are lethal (Meyer et al. 2000, Zizioli et al. 1999).

**Human Diseases**

As might be expected, there are many human diseases associated with defects in CCV formation. In fact studies of familial hypercholesterolemia by Anderson et al. (1977) provided a key observation that contributed to defining the role of CCVs in receptor-mediated endocytosis. In one form of this disease, the recognition signal for AP2 in the cytoplasmic domain of the low-density-lipoprotein receptor is mutated, leading to a lack of endocytosis and accumulation of low-density lipoprotein in the blood. Another metabolic disease that apparently results from altered receptor trafficking by CCVs is hereditary hemochromatosis (Enns 2001).
defective gene in hereditary hemochromatosis is a member of the extended family of molecules with homology to class I MHC molecules. This protein, called HFE, binds transferrin receptor and regulates its ability to internalize iron-loaded transferrin. The defective HFE is unable to interact with transferrin receptor, and regulation of iron metabolism is disrupted.

CCVs also play a critical role in human viral infections. CCVs are the route of entry for infection by some viruses, including influenza and Semliki Forest virus, but not others, such as polio virus (DeTulleo & Kirchhausen 1998). In the case of influenza, entry into the acidic endosome induces a conformational change in the viral hemagglutinin, causing it to become a fusion protein, which mediates viral envelope fusion and delivery of viral RNA into the cytosol. CCVs also play a role in viral pathogenesis. The HIV nef protein induces internalization of the CD4 molecules on helper T cells and causes reduction of expression of class I MHC molecules on infected T cells. Both effects appear to be part of the HIV immune evasion strategy. The reduction of class I molecules abrogates recognition of infected cells by cytotoxic T cells (Collins et al. 1998), and the reduction of CD4 expression interferes with helper T cell function. For alteration of CD4 traffic, it appears that nef functions by causing CD4 internalization in CCVs (Piguet et al. 1998). For reduction of class I MHC molecule expression, nef seems to alter class I traffic in the TGN in a PACS1-dependent pathway (Piguet et al. 2000). The exact mechanisms by which nef affects membrane traffic are still under investigation and have been the subject of much debate (Oldridge & Marsh 1998). Nef binds to a subunit of the proton pump, responsible for acidifying endosomes, which itself can bind AP2 (Lu et al. 1998). Nef has also been shown in vitro to interact directly with AP1 and, albeit weakly, with AP2 (Bresnahan et al. 1999, Greenberg et al. 1998). Another viral protein that interacts with AP1 is the E6 protein of bovine papilloma virus (Tong et al. 1998). The E6 protein is involved in transformation, and a homolog is present in human papilloma virus. Its AP1-binding activity could either be important for the virus life cycle or somehow participate in induction of cellular transformation.

A number of leukemias have associated defects in genes of proteins involved in CCV formation (Floyd & De Camilli 1998). CALM becomes fused to the AF10 gene in a translocation that occurs in both acute myeoblastic leukemia and acute lymphoblastic leukemia (Dreyling et al. 1996). Two translocations in acute myeoblastic leukemia result in fusion of Eps15 with HRX (Salcini et al. 1999). Endophilin is also fused to HRX in another case of acute leukemia (So et al. 2000). The donor gene in these two cases could give rise to a fusion protein causing transformation, as AF10 and HRX are both putative transcription factors. However, it is entirely conceivable that the endocytosis-related portion of the fusion protein could play a role in transformation because of the importance of endocytosis in growth factor receptor regulation. A number of oncogenes are constitutively activated receptor tyrosine kinases (RTKs) (Riese & Stern 1998), and expression of these receptors is regulated by CCV-mediated endocytosis. Thus CCV gene products are likely to play a tumor suppressor function. It is notable that tumor
suppression of meningioma has been associated with a gene encoding the β1-subunit of AP1 (Peyrard et al. 1994). The AP1-mediated pathway could potentially affect the presence of signaling molecules in endosomes from which RTKs can stimulate signaling pathways (Beattie et al. 2000).

As in model genetic systems, disruptions in the CCV pathways in humans are associated with neurological disorders. Histological distribution of CLCs is altered in brain tissue from patients with Alzheimer’s disease and Pick’s disease (Nakamura et al. 1994a,b). A significant reduction of expression of AP180 and AP2 in particular regions of brain from patients with Alzheimer’s disease has also been reported (Yao et al. 1999, 2000). In patients with Huntington’s disease, the huntington protein is altered by an expanded polyglutamine tract in the N terminus. Huntington has been reported to be enriched in CCVs, and Hip1R has been implicated in CCV-actin interactions (Engqvist-Goldstein et al. 1999, Velier et al. 1998). The dynamin/synaptojanin-binding protein intersectin is encoded in the Down’s syndrome region of chromosome 21 (Pucharcos et al. 1999), and its overexpression could potentially be responsible for brain defects. It is interesting that genetic defects in the human β3A-subunit of the AP3 adaptor, which occurs in some cases of Hermansky-Pudlak syndrome, do not have a neurological phenotype (Dell’Angelica et al. 1999b). Similarly to AP3 defects in M. musculus and D. melanogaster, Hermansky-Pudlak syndrome patients present with albinism because of melanosome abnormalities and have platelet storage deficiency because of defects in formation of platelet dense granules.

Muscle defects are also associated with CCV malfunction. CCVs are present in the autophagic vacuoles of patients with distal myopathy with rimmed vacuoles (Kumamoto et al. 2000). Patients with DiGeorge and/or velocardiofacial syndromes are heterozygous for a deletion in chromosome 22, which includes the gene encoding CHC22. These patients also exhibit muscle weakness and hypocalcemic tetany, characterized by muscle seizures, which occur because of lower-than-normal levels of calcium (Liu et al. 2001b). With the mapping of the human genome completed, it is certain that many more disease genes will turn out to be components of the CCV formation pathway.

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to provide links through review articles to all the outstanding work that has laid
the foundation for the recent developments we have reviewed.

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Figure 1  Intracellular location and morphology of clathrin-coated vesicles (CCV). (A) Intracellular transport steps mediated by CCV during endocytosis and secretion are delineated in blue, in comparison with the intracellular locations of other transport vesicles (other colors, as defined). (B) Distribution of CCV in a HeLa cell labeled with the X22 anti-clathrin heavy chain monoclonal antibody and fluorescent anti-immunoglobulin. (C) A membrane-associated clathrin lattice and emerging clathrin-coated pit. [Reproduced with permission from Heuser et al. (1987) and with copyright permission from Rockefeller University Press.] (D) A clathrin triskelion purified from bovine brain CCVs and visualized by platinum shadowing. (B and D) Images from our laboratory [reproduced from (Liu et al. 2001a) with the copyright permission of Oxford University Press]. The bars indicate the following dimensions: (B) 5 µm, (C) 33 nm, (D) 20 nm.
Figure 3  (See legend on next page)
**Figure 3** (See figure on previous page) Molecular structures of clathrin-coated vesicle components and regulatory proteins, as of April 2001. (For information on where each domain lies in the primary structure of the protein, see Figure 4; for information on the domain’s interactions, see Table 1.) Images were generated from coordinates deposited in the Protein Data Bank (Berman et al. 2000) using Molscript (Kraulis 1991) and Raster3D (Merritt & Murphy 1994). The colors are used to highlight different structural features. Ligands are shown in red, and red balls depict bound calcium atoms, or magnesium in the case of hsc70 and synaptotagmin. The sources for the coordinates are as follows: clathrin proximal leg PDB ID, 1B89 (Ybe et al. 1999); clathrin terminal domain with bound β-adaptin 3 peptide PDB ID, 1C9I (ter Haar et al. 2000); adaptor complex α-ear PDB ID, 1B9K (Owen et al. 1999); adaptor complex β2-ear PDB ID, 1E42 (Owen et al. 2000); adaptor complex µ2-subunit with bound TGN38 peptide PDB ID, 1BXX (Owen & Evans 1998); CALM ENTH domain with bound PI(4,5)P2 PDB ID, 1HFA (Ford et al. 2001); dynamin1 PH domain PDB ID, 2DYN (Timm et al. 1994); amphiphysin2 SH3 domain PDB ID, 1BB9 (Owen et al. 1998); synaptotagmin III C2A and C2B domains PDB ID, 1DQV (Sutton et al. 1999); Eps15 EH1 domain PDB ID, 1QJT (Whitehead et al. 1999); Eps15 EH2 domain with bound NPF-containing peptide PDB ID, 1FF1 (de Beer et al. 2000); Eps15 EH3 domain PDB ID, 1C07 (Enmon et al. 2000); epsin1 ENTH domain PDB ID, 1EDU (Hyman et al. 2000); hsc70 ATPase domain with bound ATP and Mg2⁺ ions PDB ID, 3HSC (Flaherty et al. 1990); and hsc70 substrate binding domain PDB ID, 7HSC (Morshauser et al. 1999).
Figure 4 (See legend on next page)
Figure 4  (See figure on previous page) Molecular interactions between clathrin-coated vesicle components and regulatory proteins. Clathrin and adaptors interact with myriad factors that influence assembly, vesicle budding, cytoskeleton interaction, signaling, and phospholipid interaction. The central cartoons represent clathrin and adaptors, and a bar proportional to protein length represents other proteins. Arrows represent binding interactions between proteins. Colors represent the domain location and length within the primary structure of the protein. Where two domains overlap, the two colors are shown as diagonal stripes. In cases where proteins are alternatively spliced or there are family variants, the longest member of the protein family is depicted. (Note that arrows do not necessarily indicate which domains are interacting; for that information, see Table 1). Large gray brackets indicate binding of multiple proteins, e.g., auxilin, hsc70, and Hip1R all bind to clathrin. Pink ovals enclosing PL represent phospholipid binding sites.
Figure 5  A cryo-electron microscopy image of a whole clathrin basket [reproduced from Smith et al. (1998) with permission of the authors and with copyright permission from Oxford University Press]. The image has been colored to show the location of individual triskelia, similar to the version of this image reproduced by Marsh & McMahon (1999). One side of a polygon of the clathrin lattice is composed of segments of clathrin legs from four different triskelia. Two proximal legs from the red and green triskelia make up the top layer of the lattice. The yellow distal legs of two triskelia centered at adjacent vertices in the lattice can be seen curving underneath the proximal legs to form a deeper layer of the lattice. Note that the terminal domains of the triskelia are not included in this image. The terminal domains curve into the center of the polyhedron, under the vertices (Musacchio et al. 1999).