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CLATHRIN-COATED VESICLE FORMATION AND PROTEIN SORTING: An Integrated Process

Sandra L. Schmid
Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT

Clathrin-coated vesicles were the first discovered and remain the most extensively characterized transport vesicles. They mediate endocytosis of transmembrane receptors and transport of newly synthesized lysosomal hydrolases from the trans-Golgi network to the lysosome. Cell-free assays for coat assembly, membrane binding, and coated vesicle budding have provided detailed functional and structural information about how the major coat constituents, clathrin and the adaptor protein complexes, interact with each other, with membranes, and with the sorting signals found on cargo molecules. Coat constituents not only serve to shape the budding vesicle, but also play a direct role in the packaging of cargo, suggesting that protein sorting and vesicle budding are functionally integrated. The functional interplay between the coated vesicle machinery and its cargo could ensure sorting fidelity and packaging efficiency and might enable modulation of vesicular trafficking in response to demand.

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INTRODUCTION

Most, if not all, of the small vesicles that transport transmembrane and lumenal proteins between organelles in eukaryotic cells are initially encased in a proteinaceous "coat." These coats, in general, assemble from multiple hetero-oligomeric complexes derived from the cytosol. They are believed to be involved in both physically forming the transport vesicle and selectively packaging its cargo. Four sets of coat protein complexes, each mediating different transport steps, have been identified and functionally characterized. Two sets of coat proteins contain clathrin: One, along with AP2 (adaptor protein, also called assembly protein) complexes, drives endocytic vesicle formation at the plasma membrane (PM); the other, along with AP1 complexes, drives transport vesicle formation at the trans-Golgi network (TGN). A third protein complex called coatomer assembles together with ADP-ribosylation factor (ARF)-1 to form so-called COPI vesicles that mediate retrograde transport within the Golgi and between the Golgi and the endoplasmic reticulum (ER) (3–5). The fourth set of coat proteins, which includes the sec23p/24p complex, the sec13/31p complex, and sar1p, assemble to drive COPII vesicle formation at the ER (6, 7). Other clathrin- and non-clathrin-containing coat complexes that mediate other transport events have been (8–12) and will continue to be identified as other vesicular trafficking pathways are dissected.

Transport vesicle formation is a multi-step process. First, the soluble constituents of the coat are targeted sequentially or together to the appropriate membrane where they coassemble to demark the incipient vesicle-forming area (bud site). Cargo molecules are concentrated at this bud site, although the sequence of these events may vary; that is, cargo may recruit coat constituents, cargo may

be recruited into emerging bud sites, or cargo and coat may be recruited coordinately. Recent biochemical and genetic studies have identified specific coat protein subunits that interact directly with sorting signals on cargo molecules and receptors, ensuring the efficient and selective packaging of the cargo into the newly emerging transport vesicle (4, 13). Thus, interacting subunits of the coat complex both trigger vesicle budding and package cargo into the vesicle. A coated vesicle is formed when the emerging bud detaches from the membrane. In some, but not all cases, coat assembly may be all that is needed to drive vesicle detachment in vitro. However, coated vesicle formation and the accurate packaging of cargo in vivo are likely to require other regulatory factors (14–16). For example, clathrin-coated vesicle budding from the PM and TGN requires a member of the dynamin family of GTPases (17, 18). Little is known about the membrane fusion or scission event, initiated on the outer leaflet of the membrane, that is required to complete vesicle formation. The last step in the production of a transport-competent vesicle is the uncoating reaction, which serves to release the vesicle for fusion with its target organelle.

COPI- and COPII-mediated vesicle formation have been extensively studied in vitro, and substantial insight into the roles of various COP constituents has been gained from both biochemical and genetic analyses. Results from these studies have been the focus of several recent reviews (5, 7, 19, 20). However, understanding of the structure of the COPI and COPII coats and of the subunit interactions required for coat assembly and cargo packaging is in its infancy. COPI-coated vesicles were first identified in 1989 (21). COPII-coated vesicles were isolated from yeast in 1994 (6) and have only recently been isolated from mammalian cells (14). In contrast, clathrin-coated vesicles, described as vesicles in a basket, were first isolated more than 25 years ago by Kanaseki & Kadota (22). Since then, all of the major constituents of the clathrin coat have been purified and the corresponding genes cloned. Moreover, structural and biochemical studies have provided high-resolution models for coat assembly. Finally, in vitro assays for coat-protein interaction and self-assembly, as well as for coated vesicle formation from membranes, have been developed. Some of these subjects have been reviewed relatively recently (1, 2, 23). This review focuses on the many new insights that have been gained about the structure and function of clathrin-coat constituents and about the mechanisms of clathrin-coated vesicle budding and protein sorting.

CONSTITUENTS OF THE CLATHRIN COAT

Clathrin and Adaptor Protein Complexes Coassemble to Form the Coat

Biochemical characterization of preparations of highly enriched coated vesicles from a variety of tissue sources led to the identification of the major coat

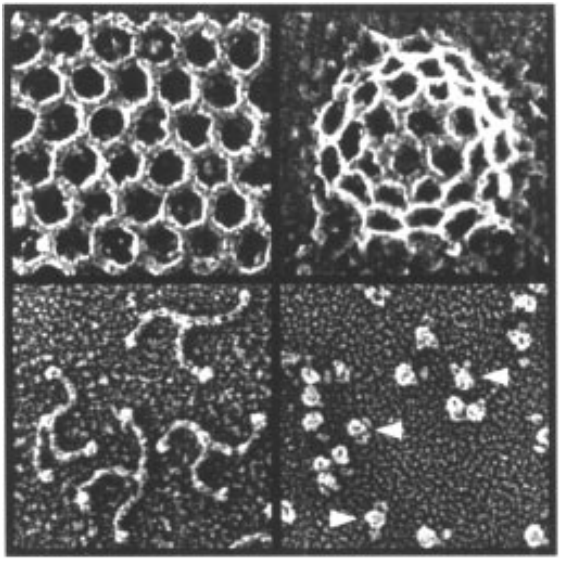

Figure 1 Clathrin-coated pits and their constituents. Clathrin triskelions (*bottom left*) and AP2 complexes (*bottom right*) are visualized by platinum rotary shadowing. The top panels show an area of a flat clathrin lattice (composed exclusively of hexagons) and a curved coated pit (in which pentagons have been incorporated) visualized by quick-freeze deep-etch electron microscopy. The images are shown to matching scale, at ∼250,000X magnification. Figure courtesy of John Heuser, Washington University, St. Louis.

constituents as two oligomeric proteins, clathrin and AP complexes (24–27). Clathrin consists of three 192-kDa heavy chains (HCs) each bound to either of two ∼30-kDa light chains, LCa or LCb. This complex is called a triskelion, based on its three-legged appearance when viewed by negative stain or rotary shadowing (see Figure 1) (28, 29). Triskelions are the assembly units of the polygonal lattice composed of hexagons and pentagons that demarks the bud site and eventually encases the transport vesicle. Each triskelion leg is comprised of an extended HC molecule oriented with its C terminus at the vertex (Figure 2). The central hub of a triskelion contains three regions: a small globular domain at the extreme C-terminus, a trimerization domain that constitutes the vertex, and a proximal leg, to which the LCs are bound. The distal leg segment and the globular, ∼50–kDa terminal domain located at the N terminus of each HC are connected to the hub through a protease-sensitive bend, also called a knee. Clathrin triskelions are structurally heterogeneous because the distribution of the two LCs among them is random (30). The functional significance, if any, of this heterogeneity remains obscure. Yeast has only one LC (31), and some mammalian cell lines appear to express only one LC (32).

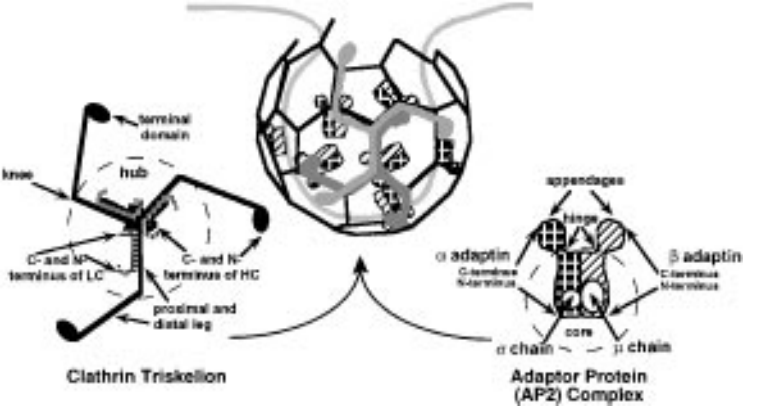

Figure 2 Structure of clathrin triskelions and AP2 complexes and a model for coat assembly. The domain structures of clathrin and AP complexes as defined by limited proteolysis are shown. The HC of clathrin is shown to fold back on itself at the region where it interacts with LCs. The μ and σ subunits of AP are shown to interact with the β and α chains, respectively, and with each other. This model is based on subunit interactions mapped using the two-hybrid system and on the finding that $\sigma 1$ chains codistribute with $\mu 1$ chains and interact with N-terminal sequences on γ, while $\sigma 2$ codistributes with $\mu 2$ and interacts with N-terminal sequences on α (45). Within the coated pit, APs are shown to interact with the membrane via their core regions (70), placing the μ chains in proximity with receptor tails. Sites for AP-clathrin interactions have been identified on both the β and α subunits of APs and on both the terminal domain and hub regions of clathrin. The placement of APs within the coated pit reflects this information, but for simplicity, only a few APs are illustrated. See text for details.

In weakly acidic, Ca^{2+}-containing buffers of low ionic strength, clathrin triskelions spontaneously self-assemble, forming a heterogeneous population of closed polyhedral structures called cages (27, 33, 34). Analysis of these clathrin cages by high-resolution electron microscopy (EM) led to the current structural model for clathrin-clathrin interactions required to form the polyhedral basket that surrounds a coated vesicle (28, 35, 36). In this model (Figure 2), a triskelion lies at the center of each vertex of the polygon, each leg extending along two edges of the polygon with the knee located at the adjacent vertex. In this way, each edge is composed of two antiparallel proximal legs (with associated LCs) extending from adjacent vertices, and two antiparallel distal legs extending from the next neighboring vertices along the lattice. The terminal domain appears to extend toward the center of the cage. According to this model, similar protein-protein interactions are involved in both hexagon and pentagon assembly, a principle of quasi-equivalence also employed in the assembly of viral coat protein subunits (37, 38).

The second major coat constituents, the APs, were first defined functionally by their ability to promote clathrin assembly under more physiological conditions (1). AP complexes coassemble with clathrin to form “coats” of uniform

size that more closely resemble those on coated vesicles (Figure 1). At least two major AP complexes, distinguished by their subunit composition, are ubiquitously expressed. AP1 and AP2 are structurally similar, consisting of two distinct high-molecular-weight subunits of ~100 kDa (generally called adaptins), two medium-size subunits (47–50 kDa), and two small subunits (17–19 kDa). Several laboratories have contributed to the molecular and biochemical characterization of AP1 and AP2 subunits (1, 2, 39–41), hence their nomenclature varies. AP2 complexes consist of α and $\beta 2$ (also called β) adaptins, a 50-kDa subunit referred to as AP50 or $\mu 2$ (for medium chain of AP2), and a 17-kDa subunit referred to as AP17 or $\sigma 2$ (for small chain of AP2). Similarly, AP1 complexes consist of γ and $\beta 1$ (or β') adaptins, AP47 (or $\mu 1$), and AP19 (or $\sigma 1$).

The Greek letter designations for AP1 and AP2 subunits connote structural (and supposedly functional) similarity (42, 43). Thus, α and γ adaptins are most divergent (~30% amino acid sequence identity, restricted to the N-terminal region), whereas the respective μ and σ chains of AP1 share considerable (~50%) homology with their respective μ and σ chains in AP2. $\beta 1$ and $\beta 2$ adaptins are highly homologous (>90%) and may in fact be functionally interchangeable (44, 45). Because the molecular weights of AP constituents may vary among species and even tissues, the Greek designator (indicating sequence relationships) and the numerical designator (indicating the AP complex to which each subunit associates) are used throughout this review. New AP complexes and additional large, medium, and small chains of similar molecular weights have been identified (11, 12, 46). Thus, an advantage of this nomenclature is that it can be readily expanded to include newly identified AP complexes and subunits (for example, a putative AP4 complex might contain a δ and β adaptin, along with $\mu 4$ and $\sigma 4$ subunits).

When viewed by platinum-shadowing (47), AP complexes appear to have two small appendages connected to a brick-shaped core region via a flexible stalk that is protease sensitive (Figure 1). Limited proteolysis removes the appendages, which are released as ~30-kDa domains from the C terminus of the α (or γ) and $\beta 2$ (or $\beta 1$) adaptins. The protease-resistant μ and σ chains remain associated with the ~60–65-kDa core domains of the adaptin subunits (see Figure 2). Analysis of functions retained by the products generated by proteolytic digestion of both clathrin and AP complexes have contributed greatly to current biochemical and structural understanding of these molecules.

STRUCTURE AND FUNCTION OF CLATHRIN

Given the ability of clathrin to spontaneously self-assemble into cages and coats, it is generally assumed to play a major role in deforming the underlying

Table 1 Functionally defined domains of clathrin

Heavy chain	Residues	Function	References
C-terminal Pro/Gly-rich domain	1615–1675	Unknown	50, 207
Trimerization domain	1490–1587	HC trimerization	48, 49
Proximal leg	~1075–1675	LC binding Cage assembly AP2 binding? Hsc70 binding	48, 49
Distal leg	~523–1073	Cage assembly AP2 binding?	48, 207
Terminal domain	1–~ 479	AP2 binding Hsc70 binding β-arrestin binding	56, 207 JH Keen (personal communication)
Light chain			
Heptad repeats	109–158	HC binding	39
	ser11, 13 (LCb)	Phosphorylation sites	23
	85–96	Ca^{2+}-binding sites	23
	41–71 (LCa)	Hsc70 interaction	171

membrane to form the budding vesicle. However, accessory proteins that serve to target and coordinate clathrin assembly and disassembly are essential for its function in coated vesicle budding. Some of these accessory proteins have been identified. AP complexes coordinate assembly; auxilin and hsc70 (the uncoating ATPase) regulate clathrin disassembly (see below). Extensive biochemical analysis has provided a detailed map of functional domains in the clathrin HC involved in its interactions with the LCs, with other clathrin molecules (during self-assembly), and with APs. These data are summarized in Table 1 and discussed below.

Clathrin Domains Required for Triskelion Assembly

Regions of clathrin required for trimerization have been defined by limited proteolysis (48) and, more recently, by expression of clathrin HC fragments in *Escherichia coli* (49). The minimal trimerization domain has been mapped by limited proteolysis to residues 1490–1587 in the HC (48). This region includes part of a predicted α-helix between residues 1522 and 1572 (48). Although the structural basis for clathrin trimerization is not known, constructs consisting of residues 1521–1589 of the clathrin HC fused to maltose binding protein are trimeric when expressed in *E. coli* (49). The Pro-Gly–rich C-terminal ~70–80 residues are not required for trimerization; instead, they appear to form a small

globular domain of unknown function, underlying the vertex. This domain is apparently not essential for clathrin function in yeast, because expression of C terminally truncated clathrin rescues a clathrin HC-deficient mutant (50). HC trimerization is defective in yeast lacking the LC, suggesting that LC may play a role in the assembly and/or stability of clathrin trimers in vivo (51).

Assays in which dissociated light chains bind back to HCs have been used in conjunction with limited proteolysis and antibody inhibition to map the regions on both polypeptides involved in their interactions (30, 52, 53). HC-LC association has also been studied in vivo in mammalian cells and in *E. coli* expressing various LC or HC deletion mutants (32). HC is believed to interact with LC through a coiled-coil comprised of a four-helix bundle. Because the two sets of predicted α-helices contributed by the HC are well spaced (residues 1178–1151 and 1460–1489), current models suggest that the HC folds back on itself in this region (Figure 2). LCs bind with their C terminus oriented toward the triskelion vertex through a central domain that encodes two tracts of heptad repeats interrupted by a predicted turn (30, 52–54). The break in the heptad repeats, together with the observation that antibodies directed toward epitopes at the N- and C-terminal ends of this central region compete with each other for binding to LCs when associated with HCs (48), suggested that the LC might also fold back on itself. However, high-resolution immunolocalization studies using antibodies directed against the N terminus of LCs have provided evidence for the extended conformation shown in Figure 2 (54). If the LCs did fold back on themselves, then they would extend only ~6 nm along the length of the proximal leg. However, N-terminal epitopes were localized 10–13 nm from the triskelion vertex (54), whereas C-terminal epitopes were localized to the vertex. The finding that LCs bind only to trimerized HCs (48) suggests that LC-HC interaction determinants might also extend into, or at least be influenced by, the HC trimerization domain. Verification of this model will require three-dimensional structural analysis of HC-LC complexes, a possibility now accessible given the recent success in coexpressing and assembling homogeneous HC-LC complexes in *E. coli* (49).

Clathrin Domains Required for Cage Assembly

Limited proteolysis of assembled clathrin cages removes the LCs and generates so-called truncated triskelions containing an ~90-kDa HC fragment corresponding to the triskelion hub and portions of the distal leg. These truncated triskelions retain the ability to bind LCs and to reassemble into cages after dissociation (55, 56). More extensive proteolysis of isolated clathrin HCs defines a fragment of 68 kDa as constituting the minimal hub domain (48). When expressed and purified from *E. coli*, this domain (residues 1075–1675) forms trimers that can self-assemble into cages (49). The resulting cages are,

however, considerably larger and more irregular than those formed by either intact or N-terminally truncated triskelions, suggesting that more distal regions of the leg play important roles in establishing the correct supramolecular contacts in the cage. LCs are not required for cage assembly in vitro, but their presence confers both pH-sensitivity and cation-dependence to the clathrin assembly reaction (49, 57). LCs are subject to phosphorylation in vivo, and they bind both Ca^{2+} and calmodulin in vitro (23, 39, and references therein). Thus, LCs are believed to serve as regulatory subunits that affect both clathrin assembly and disassembly.

Clathrin Domains Required for Interaction with Adaptor Proteins

Insight into clathrin-AP interactions first came from a comparison of the structure of cages composed solely of clathrin with that of coats containing both clathrin and AP complexes (36). These studies showed that APs are clustered in a central core surrounded by the polygonal lattice formed by the HC hub and distal legs (Figure 2). The terminal domains of HCs extend inward from the lattice to interact with the central AP core. Several assays have been developed to study AP-clathrin interactions in vitro. One approach involves assessing the ability of APs to bind to immobilized clathrin or clathrin fragments. Another approach follows the ability of APs to bind to and cosediment with preassembled clathrin cages. A third approach tests the ability of APs to coassemble, under physiological conditions, with soluble clathrin or clathrin fragments to form sedimentable cages. A fourth and less direct approach measures the binding of clathrin or clathrin fragments to cell membranes, because this association is believed to be mediated by clathrin-AP interactions.

These biochemical analyses have provided evidence for two binding sites on the clathrin HC for interaction with APs: one on the proximal leg or hub, and a second on the terminal domain (58, 59). APs are able to bind directly and independently both to truncated triskelions that are either assembled into "clipped" cages or immobilized on Sepharose and to isolated terminal domains immobilized on Sepharose (58). AP interactions with triskelion hubs are not dependent on LCs. Truncated triskelions that lack portions of the distal leg and the terminal domain retain their ability to bind to membranes, presumably through interactions with APs (60), although the possibility that binding occurs through assembly with residual intact clathrin molecules on the membranes cannot be ruled out in this case.

Functional AP2-clathrin interactions appear to require both binding sites on the clathrin triskelion. For example, APs are unable to stimulate the assembly of truncated triskelions that lack terminal domains (JH Keen, personal communication), suggesting that both sites are necessary for AP-promoted clathrin

assembly. Similarly, the interactions of AP2 with assembled clathrin cages can be qualitatively distinguished from AP interactions with unassembled triskelions or individual clathrin domains. For example, AP2-promoted clathrin assembly and AP2 interactions with either unassembled intact triskelions, truncated triskelions, or terminal domains are disrupted in the presence of 200 mM tris-hydroxymethyl aminomethane (Tris) or 5 mM polyphosphates (59, 61). In contrast, AP2 interactions with clathrin cages are resistant to Tris and polyphosphates under these conditions. The finding that AP2 interactions with clipped cages that lack terminal domains are also sensitive to Tris and polyphosphates (59) suggests that both binding sites in the assembled cage are necessary to stabilize clathrin-AP interactions. Interestingly, Hsc70, the uncoating ATPase, also appears to interact with clathrin HC both at the terminal domain and within the hub region (62). It has been proposed, therefore, that the mechanism of uncoating might involve disruption of AP-clathrin interactions, which could destabilize clathrin-clathrin interactions under physiological conditions (44).

Although the exact mechanisms of clathrin assembly and disassembly in vivo remain to be determined, the structural information discussed above and summarized in Table 1 provides a useful foundation upon which to build and test models. Mutagenesis studies in yeast (41) and in mammalian cells, as well as further development of cell-free assays that faithfully reconstitute these events, may provide the means to elucidate the mechanisms that regulate clathrin coat assembly.

STRUCTURE AND FUNCTION OF ADAPTOR PROTEIN COMPLEXES

AP complexes play multiple roles in controlling coated vesicle formation (1, 2). They are selectively targeted to and demonstrate saturable binding to specific cellular membranes: AP1 complexes bind principally to the TGN, whereas AP2 complexes are principally targeted to the PM. Upon binding, AP complexes trigger assembly of a clathrin lattice. APs also interact directly with the cytoplasmically exposed sorting signals on transmembrane proteins and presumably play a direct role in concentrating cargo molecules into coated pits. Finally, AP complexes are subject to regulation in vivo and/or in vitro by such factors as pH, inositol-polyphosphates, phosphorylation/dephosphorylation, and GTPases.

Considerable progress has been made in identifying the AP subunit(s) responsible for the various functional properties of APs (Table 2). As for clathrin, initial mapping of the domain structure of AP complexes was achieved by analysis of the products of limited proteolysis using assays for measuring clathrin-AP (described above) and AP-membrane interactions (see below). An additional advance in elucidating the structure and function of individual AP subunits was

Table 2 Functionally defined domains of adaptor proteins

Subunit (region)	Residues	Function	References
α-adaptin			
N-terminus	5–80	Inositolpolyphosphate binding	83
N-terminus	132–350	PM targeting (major) $\sigma 2$ chain interaction	45
Core region		Self-association, σ chain binding	45, 64
Hinge region		Phosphorylation site	122
Appendage	701–938	Dynamin binding	93
Appendage		PM targeting (minor)	72
Unmapped		pH-sensitive clathrin binding?	64
γ-adaptin			
N-terminus	132–331	TGN-targeting $\sigma 1$ chain interaction	45
Unmapped		Clathrin binding?	64
$\beta 1$ and $\beta 2$ adaptin			
Hinge region	616–633	Clathrin binding	44
Core region		μ chain binding	45
Appendage	704–822	TGN targeting (minor)	72
$\mu 1/\mu 2$			
		Tyrosine-motif binding/cargo recognition	13
		Membrane targeting?	45
$\sigma 1/\sigma 2$		Membrane targeting?	45

development of methods to dissociate intact AP complexes by denaturation in guanidine-HCl and to renature isolated subunits, which retain their ability to interact with clathrin (63). Extension of these methods to renaturation of AP subunits expressed in *E. coli* (42, 44) and to AP subunits prepared by in vitro transcription translation (64) now provides an opportunity for even more refined biochemical analysis. Given the sequence homologies and apparent structural similarities among the various subunits that constitute AP1 and AP2 complexes, it is generally assumed that they are functionally homologous, although they mediate distinct sorting events. This assumption has held for all homologous subunits from AP1 and AP2 complexes that have been examined (for example, α vs γ, $\beta 1$ vs $\beta 2$, $\mu 1$ vs $\mu 2$, etc).

Adaptor Protein Domains Required for Clathrin Binding and Coat Assembly

The exact mechanism of AP-promoted clathrin assembly is not known, but it is believed to involve multivalent interactions between clathrin and APs, either at distinct binding sites within the same AP complex (65) or at a single binding

site within self-associated AP complexes (44), or both. Either alternative can be accommodated by the two AP binding sites that have been identified on clathrin (see above). These models can be distinguished, however, by identifying the number and location of clathrin binding sites on APs. Limited proteolysis cleaves intact AP complexes within the flexible hinge region separating the appendage domains from the AP core (Figure 2). Neither AP cores alone nor appendage domains are able to support coat assembly. When proteolytic digestion is performed on assembled coats under buffer conditions that minimize AP-AP interactions, both the appendages and the AP cores dissociate from the cages, coincident with cleavage of the β subunit (66). These results suggested that stable association with clathrin requires interactions mediated by both the appendage and core domains of β-subunits. Alternatively, the hinge region of β, which is cleaved during proteolysis, may also contribute some essential contacts.

Confirmation of a role for β adaptin in clathrin binding came from studies showing that recombinant $\beta 1$ and $\beta 2$ adaptins, expressed and renatured from *E. coli*, support coat assembly in vitro (42). Analysis of individually expressed fragments of β adaptins revealed that both a trunk/hinge fragment and an appendage domain/hinge fragment supported coat assembly. N- and C-terminal deletion mutagenesis of these fragments localized this assembly activity to the hinge region (44). While these fragments can promote clathrin assembly, it is important to note that their efficacy relative to intact AP complexes (and to each other) has not been established. For example, assembly activity was only demonstrated to occur at pH 6.4, whereas AP-promoted clathrin assembly occurs at physiological pH. Nonetheless, these results provide some suggestive evidence that a principal (and perhaps the only) determinant for assembly activity may reside in a conserved region (residues 616–663 of $\beta 2$) within the otherwise variable hinge domain of β adaptins. Coat assembly promoted by either the N-terminally or C-terminally derived hinge-containing fragments is believed to require their self-association, on the basis of the high protein concentrations required and the finding that neither fragment interacts stably with clathrin (44). One further caveat in interpreting these data, which are derived using proteins renatured from *E. coli* inclusion bodies, is that myelin basic protein (MBP) also displays coat assembly activity in vitro (67). The effect of MBP raises the possibility that even nonspecific, multivalent interactions may be able to induce clathrin assembly under in vitro assay conditions. However, some additional support for the role of the appendage/hinge region of $\beta 1$ in AP-clathrin interactions comes from membrane binding assays in which only the intact AP1 complexes efficiently recruit clathrin despite the fact that both intact AP1 complexes and AP1 cores lacking the hinge/appendage domains bind to isolated Golgi membranes (68).

The finding that self-association of a single binding site within the hinge region of β adaptins appears sufficient, under some conditions, to promote clathrin assembly supports a model in which coat assembly is initiated by the recruitment of clathrin to AP complexes already clustered on the membrane (44). This model does not require either two AP binding sites on clathrin triskelions or two clathrin binding sites on APs. Instead, it suggests that clathrin assembly continues by the cooperative addition of clathrin triskelia (through clathrin-clathrin interactions) at a nucleation site. Dual sites for clathrin-AP interactions have, however, been identified not only on clathrin, but also APs. α-adaptins generated by in vitro transcription-translation can bind directly to clathrin cages (64), although they appear unable to stimulate clathrin assembly. While AP2-clathrin interactions show only a slight pH-dependence, the binding of α-adaptins, β-adaptins, and presumably β-adaptin fragments (see above) to clathrin is substantially reduced at physiological pH. Thus, functional and physiological interaction of AP2 with clathrin is likely to involve multiple contact sites. For example, the α-adaptin may directly interact with one of the two AP-binding sites on the clathrin triskelion to cooperate with the β-adaptin in promoting clathrin assembly in vivo. The binding site(s) on clathrin for interaction with either isolated α-adaptins or β-hinge regions have not been identified. Another possibility is that the α-subunit plays an indirect role in coat assembly in vivo by promoting AP2 self-assembly and concomittantly the clustering of β-adaptins. The α-adaptin–containing AP2 complexes, but not the γ-adaptin–containing AP1 complexes, show a high propensity for self-association under physiological conditions (69). Further analysis of the assembly properties of deletion and point mutants of various adaptin subunits studied in the context of AP complexes should provide valuable insights into their respective roles in coat assembly.

Adaptor Protein Domains Required for Membrane Binding and Targeting

In vitro assays using intact and proteolytically cleaved AP complexes have established that membrane binding is mediated principally by the cores of the AP1 and AP2 complexes (68, 70). In vivo, AP1 complexes are targeted efficiently to the TGN, whereas AP2 complexes bind to the PM. Since the β-chains are highly conserved and appear to play a role in AP interactions with clathrin, it seemed likely that the more divergent α and γ subunits of APs might play a role in membrane targeting. Consistent with this hypothesis, isolated α-subunits, but not β-subunits, are able to bind to PM (71). Given the high degree of structural conservation between the α and γ subunits, a series of chimeras was generated and expressed in mammalian cells. The first of these studies (72), in which the C-terminal appendage and hinge domains were exchanged between α and γ adaptins, established that the major membrane

targeting determinants resided within the N-terminal core region. More extensive analysis defined a 200-residue region within the N-terminal core domain of both α and γ adaptins, which was necessary for their efficient targeting to either the PM or the TGN, respectively (45). In each case, however, a larger fraction was found in the soluble pool, indicating that none of the chimeras or mutants were able to assemble onto membranes as efficiently as the corresponding wild-type subunit. This altered distribution could reflect impaired folding, less efficient AP complex assembly, or a requirement for additional targeting information residing elsewhere in these polypeptides.

An important and unexpected finding from these studies was that localization of the AP complexes correlated to a switch of their μ and σ chains (45). That is, those α/γ-chimeras that coassembled with $\mu 1$ and $\sigma 1$ subunits were invariably targeted to the TGN, whereas those α/γ-chimeras that coassembled with $\mu 2$ and $\sigma 2$ subunits were invariably targeted to the PM. Interestingly, in the same assay, $\beta 1$ and $\beta 2$ adaptins showed promiscuity, and both could be detected with each of the α/γ chimeras. The yeast two-hybrid system was employed to confirm these binding specificities and to extend analysis of subunit interactions (45). It was found that both β adaptins could interact with either α or γ adaptin and with either $\mu 1$ or $\mu 2$ subunits. In contrast, $\sigma 1$ appeared to interact specifically with the N terminus of γ, whereas $\sigma 2$ interacted specifically with the N terminus of α. These results provide indirect evidence that the small and medium subunits might play a direct role in intracellular targeting of AP complexes. Interestingly, more mammalian σ and μ family members have been identified in data bases by sequence homologies than have adaptin subunits (43, 46), suggesting that heterogeneity in these smaller subunits might be responsible for targeting adaptin assembly to distinct cellular membranes. Immunolocalization of distinct σ and μ family members, together with mutagenesis studies, will be required to test this possibility.

Adaptor Protein Domains Involved in the Recognition of Sorting Signals

The finding that APs mediate clathrin assembly on authentic biological membranes, together with the well-established observation that many transmembrane receptors are concentrated in clathrin-coated pits, led to the speculation (73) that the AP complexes might act as connectors, bridging both receptors and clathrin to affect sorting. This hypothesis was tested and confirmed in an early series of experiments demonstrating that AP2, but not AP1, bound specifically to immobilized fusion proteins encoding functional endocytic sorting motifs (74, 75). Correspondingly, AP1 complexes bound to the cytoplasmic domain of the mannose-6-phosphate (M6P)-receptor, a molecule carried in TGN-derived coated vesicles. However, the affinity of APs for receptor tails was rather low,

and it was several years before these results could be reproduced (76). It is now clear, however, that AP2 complexes can interact directly with sorting signals, such as the Tyr-containing sorting motifs present both on constitutively internalized receptors and in at least one ligand-activated receptor-tyrosine kinase, the epidermal growth factor receptor (EGF)-R (77, 78). Use of limited proteolysis established that interactions with sorting sequences occur through the core region of AP2 and not through its hypervariable appendage (76, 77).

Recently, a yeast two-hybrid screen in which a triple repeat of the internalization/Golgi retrieval motif of the protein TGN38 was used as "bait" resulted in the "capture" of the $\mu 2$ subunit of AP2 (13). Subsequent analysis using the yeast two-hybrid system and direct biochemical measurements established that both the $\mu 1$ and $\mu 2$ subunits can specifically interact with a variety of Tyr-containing sorting motifs (13, 79, 80). Other subunits of AP complexes failed to interact with sorting signals using the two-hybrid system (79). Together these findings establish a role for the μ subunits in cargo recognition.

Adaptor-Protein-2 Complexes Bind Inositol Polyphosphates

AP2 complexes bind inositolpolyphosphates (PPIs, such as 1,4,5-IP_3 and IP6) specifically and with high affinity (K_d = 0.1–5.0 μM, decreasing with the number of phosphates) (61, 81, 82). At similar concentrations, IPPs inhibit AP2 self-assembly, AP2-promoted clathrin assembly (69), and AP2-membrane binding (71). It has also been shown that AP2 binds polyphosphinositides that carry short chain fatty acids, such as diC_8-PtdIns-3,4-P_2 and diC_8-PtdIns-3,4,5-P_2 (PI-3,4-P_2 and PIP_3, respectively), with higher affinity than IPPs bearing the same charge (83), suggesting that the glycerol moiety and/or fatty acyl chains may enhance binding affinity. Interestingly, compared to free AP2, AP2 coassembled with clathrin displays several-fold higher affinity for PIP_3 and a correspondingly diminished affinity for IP_6 (83). Finally, the binding site for IPPs maps to the N terminus of α-adaptin; a fusion protein encoding residues 5-80 of α-adaptin showed the same binding properties as intact AP2 (83). This location is close to the region of α-adaptin involved in membrane targeting (45). Given that polyphosphoinositide lipids are integral membrane constituents, these results suggest a potential role for PIP_3 (or PI-3,4-P_2) in AP2-membrane interactions. In this regard, a PI-3 kinase (Vps34p) has been implicated in clathrin-dependent vacuolar protein sorting in yeast (84; see below); however, it has not been demonstrated that AP1 can bind IPPs. Similarly, PI-3 kinase activity has been implicated in trafficking of the receptor for platelet-derived growth factor (PDGF-R) following internalization but does not appear to be required for its clathrin-mediated endocytosis per se (85). Although considerable indirect evidence supports a role for polyphosphoinositides in regulating coated vesicle formation (86), further studies are needed to confirm this possibility.

Assembly Protein 180, a Neuron-Specific Assembly Protein

Despite its many names, AP180 (also referred to as AP3, NP185, and F1-20) bears no structural resemblance or sequence similarity to either AP1 or AP2 complexes or to any of their subunits. Neither is its mass 180 kDa. AP180 is an ~91-kDa monomer (87, 88) that migrates aberrantly on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), owing largely to a highly acidic central domain (87) and, in part, to posttranslational modification by O-linked carbohydrate (89). Although the function of AP180 in neurons is not known, in vitro it displays the highest specific activity in clathrin assembly of any known assembly protein (90). Like AP2 complexes, AP180 binds IPPs with high affinity, and IPPs inhibit AP180-promoted clathrin assembly (91). AP180-promoted clathrin assembly does not require LCs, and AP180 can compete with AP2 for binding to intact cages (90). However, unlike AP2, AP180 will not bind to proteolyzed cages or to immobilized clathrin fragments (58). These data suggest that AP180 and AP2 interact with clathrin at distinct but perhaps overlapping sites. The N-terminal 58 kDa segment of AP180, which includes most of the central acidic domain, has been expressed in *E. coli* and has full clathrin assembly activity (92). Smaller regions of AP180, both within and C-terminal to the 58-kDa N-terminal fragment bind to clathrin but are unable to promote assembly. Interestingly, the IP_6-binding site of AP180 was localized to the C-terminal region, suggesting that this domain may modulate the function of the N-terminal assembly domain (92).

Remaining Structural Issues

Work over the past several years has begun to unravel the structural and functional complexity of the major coat constituents of clathrin-coated vesicles (summarized in Tables 1 and 2). However, much remains to be learned: (*a*) How and in what way(s) do LCs regulate clathrin assembly in vivo? (*b*) What is the function of the C-terminal appendage domains of the α, γ, $\beta 1$, and $\beta 2$ adaptins? These appendages readily dissociate from and fail to interact with assembled or unassembled clathrin, are not required for membrane binding, and are apparently not involved in sorting signal recognition; yet the appendages represent the most variable domains between α and γ adaptins. Do these regions of sequence divergence encode specificity determinants or nonessential functions? Dynamin (see below) has recently been identified as a possible binding partner for the appendage domain of α adaptin, suggesting that AP2 might play a role in targeting dynamin to coated pits (93). (*c*) What is the function of the σ subunits of APs? While indirect evidence supports a role in membrane targeting, such a function has not been established definitively. (*d*) Finally, how do the various binding activities of individual subunits affect the conformation and/or function of each AP complex as a whole? Are there allosteric interactions

between subunits or between functional domains of individual subunits? How do AP-membrane interactions affect AP-clathrin interactions, and vice versa? Answers to these questions will require higher-resolution structural analysis of individual domains and complexes, as well as further functional analysis using in vitro assays that faithfully reconstitute coated pit assembly, protein sorting, coated vesicle formation, and uncoating to recapitulate the events of vesicular transport in the cell.

CELL-FREE ASSAYS FOR ANALYSIS OF COATED VESICLE FORMATION

Several assays have been developed and used successfully in examining the mechanism of coated vesicle budding (reviewed in 94–96). These assays can be subdivided into three genres: (*a*) the use of isolated organelles or membrane fractions to study coat-protein recruitment and binding, (*b*) the use of isolated organelles as the starting material for vesicle budding, and (*c*) the use of semi-intact or permeabilized whole cells to reconstitute more complex vesicular transport processes. Although each approach has advantages and disadvantages, their combined use has enabled the biochemical dissection of these complex multi-step processes. Conditions for in vitro assays are often empirically or arbitrarily determined to optimize whatever signal is being recorded by the researcher. Under these artificial conditions, normal aspects of regulation or of specificity might not occur. Therefore, it is always important to compare results from several approaches and to confirm the validity of the behavior observed by comparing them with those determined in intact cells. In this regard, genetic analysis of membrane trafficking in yeast has been particularly useful (7, 96).

Assays for Assembly of Clathrin-Coated Pits

The first assay for assembly of clathrin-coated pits used highly purified PM preparations obtained by sonicating adherent cells to remove their upper surfaces and cytosol, leaving behind exposed PM sheets (97). Endogenous coat proteins were stripped from these adherent PM sheets either by mild alkali treatment (to selectively remove membrane-bound clathrin) or by treatment with high salt (to remove both clathrin and AP2s). A series of informative experiments using this assay (70, 97–99) established that both APs and clathrin assemble from the cytosol onto saturable, high-affinity, and protease-sensitive binding sites on the PM. Purified AP2, but not AP1, complexes bound with high affinity in the absence of clathrin, and clathrin binding was dependent on bound AP2. AP2 and clathrin binding occurred at 4°C and in the absence of added nucleotides or other cytosolic factors. When assembled at this low temperature,

only flat lattices were formed. Upon incubation at 37°C, even in the absence of additional coat proteins, cytosol, or nucleotides, the flat clathrin–AP2 lattices formed invaginated coated pits. These findings led to the current model that coated-pit assembly at the PM proceeds through the specific recruitment of AP2 complexes, which in turn nucleates clathrin assembly. The use of such highly enriched PM fractions and purified coat proteins in this assay system permitted identification of functional domains involved in clathrin and AP2 interaction (see above), and provided a robust assay for identifying binding sites involved in AP2-membrane association. In fact, a 45-kDa protein fragment was obtained following limited proteolysis of isolated PM that potently inhibited the binding of AP2 to PM (70). Although this protein has not been further characterized, the finding that AP2s bind to the cytoplasmic domain of synaptotagmin in vitro has led to the suggestion that the 45-kDa inhibitor might have been derived from the cytoplasmic domain of a nonneuronal synaptotagmin isoform (100). AP2 interactions with receptor tails cannot account for the specificity of targeting to the PM (see below); thus, further studies are needed to establish the identity of proteins involved in AP2 docking at the PM.

A potential limitation of this system is that endogenous clathrin and AP2 molecules must first be chemically stripped from the membrane. This procedure could remove factors that otherwise might negatively regulate AP or clathrin binding, or could by itself leave membrane binding sites in a preactivated state. Conversely, the chemical stripping treatments might remove other facilitatory factors. This limitation is reflected in the finding that clathrin efficiently reassembles onto membrane carrying endogenous AP2s that resist the mild alkali treatment that strips clathrin, but will not assemble onto membranes that have been stripped by high-salt treatment and reconstituted with purified AP2 molecules (99). The workers who conducted this study proposed that the newly recruited AP2s could not be activated under these conditions, perhaps, as suggested above, owing to loss of essential components during high-salt stripping. Alternatively, since the clathrin used was purified after chemical extraction from isolated coated vesicles, it is possible that the fault lies in the inability to activate clathrin for interaction with newly recruited AP2 complexes. Using perforated cells (see below), purified AP2 complexes, and a clathrin-enriched cytosolic fraction supported coated-pit formation, whereas purified clathrin did not (101).

These results (99, 101) and others (see below) suggest that AP2 recruitment, which occurs at 4°C without other cofactors, is not tightly regulated. However, an alternate approach for measuring membrane recruitment of AP2 that does not involve stripping endogenous coated pits yields different results. This assay measures the assembly of immunologically distinct unfractionated AP2 complexes from unfractionated brain cytosol onto the PM of permeabilized rat

fibroblasts (102). AP2 assembly under these conditions requires ATP and is inhibited by GTPγS (103). Whether these differences reflect the use of non-treated membrane preparations, the use of unfractionated versus purified AP2, or the requirement to complete budding of endogenous coated pits before new assembly sites are made available is not known.

Assays for Budding of Coated Vesicles

Two approaches have been taken to measure coated vesicle formation in vitro. One approach, originally developed by Smythe et al (104) and later modified and extensively characterized (101, 105, 106), involves the use of semi-intact or perforated cells whose PM has been ruptured by mechanical shear. The sequestration of receptor-bound ligands into either constricted coated pits or fully sealed coated vesicles is distinguished biochemically by determining the degree of inaccessibility of the ligand to exogenously added probes (reviewed in 107). Quantitative EM confirmed that horseradish peroxidase (HRP)- or gold-conjugated ligands enter the semi-intact cells via clathrin-coated pits, then appear in coated vesicles (104, 105, 108). The sequestration of receptor-bound ligands in this in vitro system requires ATP and GTP hydrolysis (105, 106, 108), cytosolic factors including soluble clathrin and AP2 complexes (101), and interaction with internalization motifs on receptor tails (105, 109). Thus, the morphological and biochemical properties of the assay are consistent with known properties of receptor-mediated endocytosis in vivo.

Although receptor-bound ligands are efficiently sequestered into constricted coated pits in this assay, formation of fully sealed coated vesicles is considerably less efficient. Constricted coated pits are functionally defined by their ability to protect receptor-bound ligands from exogenously added macromolecular probes, but not from small, membrane-impermeant reagents (reviewed in 107). A fully sealed coated vesicle protects the ligand from both types of probes. Constricted coated pits have been detected both in vivo (110) and in vitro (105). In perforated cells, 50–60% of the total cell-associated ligands become sequestered into constricted coated pits; however, only $\sim$20–25% of receptor-bound ligands are internalized into sealed vesicles (105–107). Thus, late events in coated vesicle formation are not as efficiently reconstituted as early events, and therefore constricted coated-pit intermediates accumulate. In spite of this limitation, this assay has nonetheless been of some advantage because it facilitates dissection of clathrin-coated vesicle formation into biochemically distinct events (reviewed in 107). An assay that more efficiently and faithfully reconstitutes late events in the formation of clathrin-coated endocytic vesicles is still needed.

An alternate assay for coated vesicle budding utilizes isolated PM prepared in a manner similar to that described above for coated pit assembly assays (111); in this case, however, endogenous coated pits are not stripped from the

membrane. Coated vesicle budding is measured indirectly as the disappearance of clathrin and/or AP2 complexes from the PM fraction using an enzyme-linked immunosorbent assay. Although the disappearance of both clathrin lattices and of gold-conjugated ligands has been confirmed at the EM level, it has not been demonstrated that coated vesicles are formed or that receptors and ligands are internalized into sealed membrane compartments under these conditions. Of equal concern, many of the biochemical properties of coated vesicle formation observed in this indirect assay are not consistent with the known features of receptor-mediated endocytosis in vivo. For example, in the assay, nucleotide is required, but not ATP hydrolysis per se, and the nucleotide requirement appears nonspecific (it can be fulfilled by ADP, GTP, or ATPγS) (111). In contrast, endocytosis is severely inhibited by ATP depletion (110). Clathrin disappearance in the in vitro assay is not inhibited by GTPγS, whereas there is strong evidence that a GTPase, dynamin, is required for coated vesicle formation in vivo (reviewed in 112). Clathrin disappearance in vitro reportedly requires annexin VI and >100 μM Ca^{2+} (113), neither of which appears to be required for endocytosis in vivo (114). These disparities highlight the importance of confirming the validity of results from any in vitro assay by comparison to in vivo requirements.

MECHANISM OF FORMATION OF CLATHRIN-COATED ENDOCYTIC VESICLES

Receptor-mediated endocytosis is driven by a cycle of assembly and disassembly of clathrin-coated vesicles as shown in Figure 3. Our current understanding of the mechanism of endocytic vesicle formation, although far from complete, derives from both in vivo and in vitro analyses of clathrin coats (many discussed above) and serves as a framework for further studies.

Initiation of Coated Pit Assembly

Formation of a clathrin-coated vesicle is initiated at the PM (Figure 3, *step 1*) by recruitment of AP2 complexes to high-affinity, saturable, and protease-sensitive sites (70, 99). Although AP2 interacts directly with sorting signals on the cytoplasmic domains of PM-localized receptors, these interactions are not of sufficient strength to account for the observed tight binding. Furthermore, many receptors that recycle are more highly concentrated in endosomes than on the PM, suggesting that receptor tails are not responsible for the initial recruitment of AP2 to the PM. Based on these considerations, it is assumed that specific docking proteins for AP2 exist. The docking protein may be an isoform of synaptotagmin, a major constituent of synaptic vesicles, based on the ability of AP2 to interact with synaptotagmin in vitro (115). However, there are as yet no other functional data supporting a role for synaptotagmin

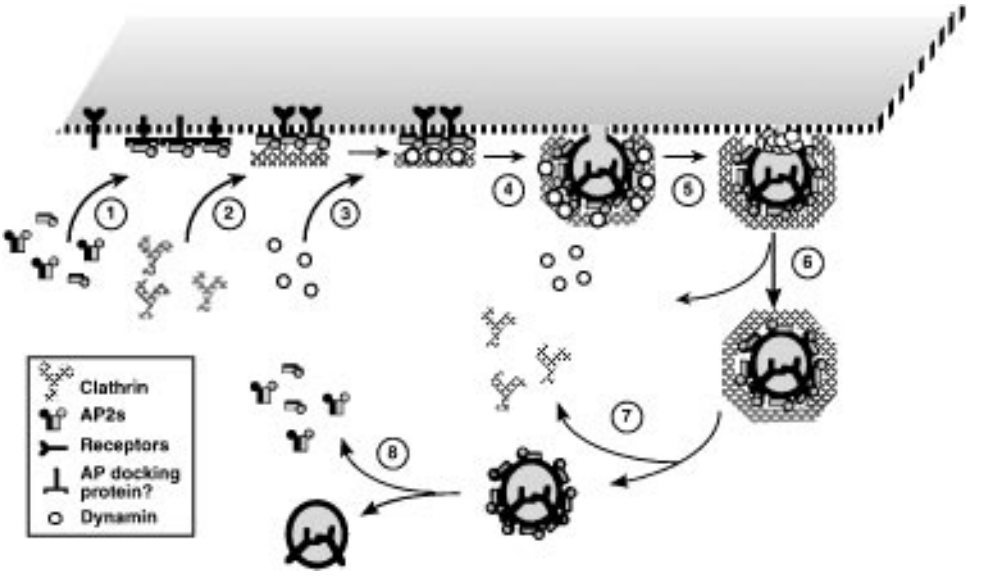

Figure 3 The clathrin-coated vesicle cycle that drives receptor-mediated endocytosis. The following steps are depicted and are described in detail in the text: *Step 1*, AP2 recruitment; *step 2*, clathrin assembly; *step 3*, dynamin recruitment; *step 4*, coated pit invagination; *step 5*, coated pit constriction requiring the redistribution of dynamin from the lattice and its assembly at the neck; *step 6*, coated vesicle budding, requiring a fusion event initiated on the external leaflet of the membrane; *step 7*, clathrin release; and *step 8*, AP2 release.

in AP2-membrane interactions. In fact, phenotypic analysis of synaptotagmin knock-out mutants in *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice (116 and references therein) point to a role for synaptotagmin as a Ca^{2+} sensor for synaptic vesicle release. On the other hand, the number of synaptic vesicles is reduced at the nerve terminals of synaptotagmin-deficient *C. elegans* (117), providing some indirect support for its role in endocytosis. However, because spontaneous synaptic vesicle release is enhanced in these animals and because the number of synaptic vesicles in the nerve terminals were measured long after formation of the synapse, the observed decrease in synaptic vesicles could be due to an enhanced rate of vesicle release (without a concomitant increase in vesicle biogenesis) or to some indirect consequence of synaptotagmin deficiency, rather than to a role for synaptotagmin in vesicle formation via endocytosis. If synaptotagmin is indeed required both for secretion and endocytosis, then perhaps more refined mutational analysis in vivo will allow separation of these two functions.

One might expect that AP2 assembly at the PM is tightly controlled, because assembly of other coats is known to be regulated. Small GTPases of the ARF and Sar family are required to trigger assembly of COPI-, AP1-, and COPII-containing coats on the Golgi, TGN, and ER, respectively (7, 16). Nonetheless, recruitment of purified AP2 complexes in vitro apparently does not require ATP, GTP, or even incubation at elevated temperatures (71, 99, 101). In contrast, recruitment of AP2 from cytosolic fractions in vitro appears to require elevated temperatures and ATP, and is inhibited by GTPγS (103, 118). Interestingly, the effect of GTPγS is to trigger mislocalized assembly of AP2 complexes (and clathrin) onto late endosomal vesicles (103). These data suggest that AP2 assembly may be subject to regulation by an as yet unidentified GTPase(s). Because purified AP2 complexes can be recruited to PM in the absence of nucleotide, these GTP-dependent regulators have not been characterized yet in vitro.

A role for the small GTPase ARF6 in endocytosis has recently been proposed (119). High levels of expression of ARF6 or an activating mutant ARF6(Q67L) inhibit transferrin receptor endocytosis in CHO (Chinese hamster ovary) cells (119) and delays receptor recycling. At high levels of expression, ARF6 and ARF6(Q67L) were localized to the PM but were not associated with clathrin-coated regions (120). In contrast, expression of a dominant-negative mutant ARF6(T27N) inhibits receptor recycling to the cell surface, but not internalization. ARF6(T27N) was localized to the endosomal compartment (120). ARF6 has not been detected in coated vesicles (121), and (in contrast to other regulatory or structural components of the endocytic machinery) it is expressed at barely detectable levels in neuronal tissues relative to nonneuronal tissues (RA Kahn, personal communication). Together, these findings are most consistent with a role for ARF6 in regulating trafficking events through the endosomal compartment in nonneuronal cells, and not in regulating clathrin-coated endocytic vesicle formation.

New insight about regulation of AP2 assembly comes from the finding that AP2 complexes in the cytosolic pool are phosphorylated quantitatively (122), whereas membrane-bound AP2 complexes are not phosphorylated. Phosphorylation occurs on the hinge region of the α and $\beta 2$ adaptins. Phosphorylated AP2 complexes are unable to bind to assembled clathrin cages (122). These results suggest that phosphorylation and dephosphorylation, by an as yet unidentified kinase(s) and phosphatase(s), may regulate AP2 interactions with the PM and/or with clathrin.

Clathrin Recruitment and Invagination of Coated Pits

After PM recruitment of AP2, and perhaps AP2 clustering via self-association (40), the docked AP2 complexes nucleate assembly of clathrin triskelions onto

the PM (Figure 3, *step 2*). As discussed above, cytosolic clathrin is a substrate for this recruitment (101), but purified clathrin extracted from coated vesicles is not (99, 101). Planar clathrin lattices can transform into curved pits in vitro (Figure 3, *step 4*) without added cytosol or nucleotides (98, 104). However, it is not certain that planar coated pits formed in vitro when assembly occurs at 4°C, or those seen on the cytoplasmic surface of adherent PM patches, are the requisite precursors to invaginated coated pits. Endocytic coated pits observed in vivo exhibit varying degrees of curvature (123), and the percentage of total clathrin-PM sites that are curved pits appears to be proportional to the rate of endocytosis (124). Therefore, it seems likely that a growing clathrin lattice gains curvature gradually during its maturation (but see 40). The shape changes required for invagination, which presumably arise from clathrin rearrangements within the lattice and/or from structural alterations during additional clathrin assembly, involve the incorporation of pentagons into an otherwise hexagonal array. How these pentagonal clathrin arrangements form is currently not understood, but several models have been proposed (125–127). Microinjection of anti-hsc70 antibodies appears to block endocytosis prior to vesicle detachment (128). These data raise the possibility that hsc70, the uncoating ATPase, may play a role in clathrin rearrangements within the lattice. Although such an activity would not be inconsistent with the in vitro properties of Hsc70 (129), its putative role in coated vesicle formation remains to be confirmed.

In perforated cells, binding of purified AP2s does not require added nucleotides or other factors (101). However, other early, as yet undefined events required for formation of coated pits that are able to sequester receptor-bound ligands do need ATP hydrolysis and are inhibited by GTPγS (101, 105, 106). The observation that lattice assembly on the membrane does not require nucleotide cofactors, whereas the sequestration of cargo into these emerging coated pits does, suggests that the nucleotide-dependent events are involved in concentrating cargo for efficient packaging and/or in coordinating coat assembly with cargo packaging. These ambiguities highlight the need to measure cargo packaging as well as formation of coated vesicles per se when assessing the biochemical requirements for endocytic transport.

Coated Pit Constriction and Vesicle Budding

Coat assembly is insufficient to drive budding of clathrin-coated endocytic vesicles, as judged by the lack of complete internalization of sequestered receptor-bound ligands. Additional cytosolic proteins, as well as ATP and GTP hydrolysis, are required in vitro for completion of at least two subsequent events. The first process is formation of constricted coated pits that remain attached to the PM via a narrow neck, but in which receptor-bound ligands are sequestered from bulky probes (Figure 3, *step 5*). Formation of constricted coated pits in

vitro is supported by GTPγS but not by ATPγS. Interestingly, this step appears to be blocked both in mitotic cells and in vitro when assays are performed using extracts from mitotic cells (123, 130). The second and final event (Figure 3, *step 6*) required for vesicle budding is membrane fission and involves both ATP and GTP hydrolysis (105, 106). The ATPases apparently required for these late stages in budding of endocytic coated vesicles have not been identified, but some evidence suggests a role for phospholipid kinases in endocytosis (86). The 100-kDa GTPase, dynamin, plays a direct role in coated pit constriction and budding of clathrin-coated vesicles (see 17, 18, 131 for recent reviews).

Dynamin, a GTPase Required for Budding of Clathrin-Coated Vesicles

Fruit flies carrying the *shibire^{ts}* allele rapidly exhibit a defect in endocytosis after shift to the restrictive temperature (131, 133; reviewed in 17). The first evidence of a role for dynamin in budding of endocytic vesicles was the discovery that mammalian dynamin was 70% identical to the *Drosophila shibire* gene product (134, 135). The role of dynamin was confirmed by the demonstration that receptor-mediated endocytosis is severely inhibited in mammalian cells transiently transfected with constructs overexpressing GTPase-defective mutants of dynamin (136, 137). Detailed morphological and biochemical examination of stable cell lines expressing these dominant-negative dynamin mutants showed that formation of endocytic coated vesicles is specifically blocked at a stage that follows assembly and invagination of coated pits but precedes formation of constricted coated pits (138, 139).

A working model for the function of dynamin in endocytosis has been proposed (140) based on experimental observations that have been reviewed elsewhere (17, 18, 141). These results include: (*a*) phenotypic analysis of stable cell lines expressing mutant dynamin (138, 139); (*b*) immunolocalization of dynamin-1 (a neuron-specific isoform) and dynamin-2 (a ubiquitously expressed isoform) to coated pits on the PM (138, 141, 142); (*c*) ability of dynamin to self-assemble into helical stacks of rings (140) that are localized to the necks of invaginated coated pits (132, 142); (*d*) in vitro interactions of dynamin with AP2 complexes (93) and the SH3-domain–containing protein, amphyphysin (143); (*e*) the role of Pro-rich SH3-domain–binding sites in dynamin in targeting dynamin to its membrane (144); (*f*) the properties of GTPase in vitro (112, 145); and (*g*) the requirements for GTP and the effects of GTP analogs on receptor-mediated endocytosis in perforated cells (106).

In this model, dynamin in its unoccupied or GDP-bound state is targeted to the clathrin lattice (Figure 3, *step 3*) through interaction with the Pro-Arg–rich C-terminal domain of dynamin. Based on in vitro binding studies, amphyphysin (or its as yet unidentified nonneuronal isoforms) is a candidate for this

targeting reaction, but the function of amphyphysin-dynamin interactions in vivo remain unknown. GTP binding triggers redistribution of dynamin from the clathrin lattice, presumably by disrupting interactions with its binding partners and allowing dynamin to self-assemble into helical rings at the necks to form constricted coated pits (Figure 3, *step 5*). These helical rings may contain a spiral consisting of as many as 20 dynamin molecules. Coordinated GTP hydrolysis could drive a concerted conformational change in the dynamin ensemble that tightens the collar and contributes thereby to the final events in coated vesicle detachment (Figure 3, *step 6*). Dynamin is less abundant, although readily detectable, on isolated coated vesicles than on coated pits (138, 142). This finding suggests that dynamin may be released from the closed vesicle after GTP hydrolysis, consistent with the finding that GTP destabilizes preassembled dynamin helices in vitro (112).

Budding and generation of fully sealed coated vesicles in vitro also requires ATP hydrolysis, indicating that dynamin function alone is insufficient to drive vesicle detachment (105, 108). The finding that a phosphatidylinositol 5-phosphatase may also be recruited to coated pits via interactions with amphyphysin (146) suggests that perhaps the ATP requirement is to generate some phosphorylated phosphoinositide derivative that might participate in these reactions. Important in this context may be the fact that dynamin contains a pleckstrin homology (PH) domain (147) that is known to bind IPPs and phosphatidylinositols in vitro (148).

Although the events and molecular mechanisms involved in formation of clathrin-coated endocytic vesicles are beginning to be elucidated at the molecular level, much remains to be understood.

CLATHRIN-COATED VESICLE BUDDING FROM THE TRANS-GOLGI NETWORK

In Vitro Assays for Coat Assembly on the Trans-Golgi Network

Assays have not yet been developed to selectively measure budding of clathrin-coated vesicles from the TGN, although other transport events from the TGN have been reconstituted (see for example 149–151, 152, and references therein). However, several groups have characterized the recruitment of AP1 complexes onto Golgi membranes (102, 118, 152–154). AP1 recruitment, unlike AP2 recruitment, appears to require ARF1 activation and is sensitive to brefeldin A. Recent data have also implicated the cytoplasmic tail of the M6P-R (the predominant cargo of lysosomally directed TGN-derived coated vesicles) as an essential component in the AP1 docking site (153). These studies are important because they implicate cargo molecules in directing coat assembly. In similar

studies, overexpression of human transferrin receptors (Tfn-R) in chick fibroblasts promoted clathrin lattice assembly on the PM (155). To ensure targeting specificity in AP1 assembly on the TGN (as for AP2 assembly on the PM), it is likely that components in addition to transmembrane cargo molecules are required. Two complementary approaches involving chemical cross-linking have provided evidence for such components. One strategy involved GTPγS-triggered recruitment of AP1 to Golgi fractions enriched from metabolically labeled cells, followed by cross-linking and identification of the labeled species cross-linked to AP1 (156). In the second method, detergent extracts of Golgi membrane preparations from metabolically labeled cells were incubated with immobilized AP1 complexes and AP1-binding proteins isolated in this fashion (157). Strikingly, both groups identified a 75–80-kDa protein (as well as others) as a predominant AP1-interacting species. Although neither group has yet been able to generate sufficient amounts of the AP1-binding protein for identification, these studies indicate that there are TGN-specific membrane proteins required to direct AP1- and clathrin-containing coat assembly.

Clathrin-Coated Vesicle Formation in Yeast

Receptor-mediated endocytosis in yeast appears to differ from that in mammalian cells in several ways: First, it is only partially inhibited in clathrin-deficient yeast strains; second, it is completely abolished by mutations in actin and in some actin-binding proteins (158, 159); and third, it appears not to involve a dynamin-related protein. With the completion of the yeast genome sequence, only two dynamin family members, Vps1p and Dnm1p, have been identified, both of which have been studied previously. Vps1p functions in protein sorting to the vacuole (160; see below), and Dnm1p functions in endosomal trafficking (161). In contrast to the situation in mammalian cells, neither dynamin-family member is required for internalization of a PM-localized receptor, the α-factor receptor. Because of these differences, yeast genetics has not yet been particularly useful for elucidating the molecular basis of endocytosis in mammalian cells (but see 41).

In contrast, most of our knowledge concerning clathrin-coated vesicle–mediated transport from the TGN is derived from genetic studies in yeast. Genetic analysis in yeast has shown that Vps1p is required for early events in vacuolar sorting (84, 160), presumably during clathrin-mediated vesicle budding. An as yet unidentified dynamin-related protein has also been localized to the Golgi compartment in mammalian cells (162). Two novel cytosolic factors, Vps34p and Vps15p, are also required for Golgi-to-vacuolar trafficking in yeast (reviewed in 84). Vps15p is a novel Ser/Thr-directed kinase anchored to the yeast Golgi membrane by a posttranslationally added fatty acyl chain. Vps15p forms a complex with Vps34p, a member of the PtdIns 3-kinase family. The

activity of Vps34p is dependent on phosphorylation by the Vps15p kinase. Whether the Vps34p/Vps15p complex is directly required for clathrin-CV formation at the TGN or is instead required for efficient concentration and sorting of vacuolar enzymes (cargo) has not been established. The effects of the PtdIns 3-kinase inhibitor, wortmannin, suggest a role for PtdIns 3-kinase activity in lysosomal trafficking in mammalian cells; however, the exact function of the phosphatidylinositol-3-phosphate–containing lipids remains unknown (163, 164).

THE UNCOATING REACTION

Recycling of clathrin from coated vesicles (Figure 3, *step 7*) is mediated in vitro by hsc70, the uncoating ATPase (165, 166). Hsc70-mediated dissociation of clathrin cages requires intact triskelions (62, 67, 167) and clathrin LCs (67, 168). ATP hydrolysis by hsc70 is required for removing the clathrin triskelion from the vesicle (165, 166), and ADP stabilizes the hsc70-clathrin complex that is released (169). Hsc70 is a member of the chaperonin family that is involved in modulating the folding state of intracellular proteins for their translocation across ER, mitochondrion and lysosome membranes, and in some cases into the nucleus (reviewed in 170). Establishing a role for hsc70 in clathrin release in vivo remains an essential goal. Toward this end, one approach takes advantage of the observation that hsc70 uncoating in vitro requires interaction with a specific domain within LCa that is lacking in LCb (171). However, neither coated vesicle–mediated transport events nor the distribution of clathrin was detectably affected in cell lines either lacking LCb (due to genetic disruption) or overexpressing LCa (32). Although these results appear to raise some question about the role of hsc70 in uncoating in vivo, the lack of apparent LC specificity may be explained by the recent identification of a cofactor, auxilin, in Hsc70-mediated clathrin release (167). Auxilin is a coated vesicle–associated protein, originally identified as a clathrin assembly protein (172), that contains a Dna-J motif. Dna-J is an essential cofactor of Dna-K, an *E. coli* hsc70 homolog (173). Recent studies have shown that auxilin contains a clathrin-binding domain, that its Dna-J motif interacts with hsc70, and that both regions are required for uncoating (174). Interestingly, when auxilin in present, hsc70-mediated disassembly of clathrin cages does not require the presence of LCs and can occur even when cages are comprised of proteolytically truncated triskelions (167).

Evidence for hsc70 function in clathrin disassembly in vivo came from microinjection studies using anti-Hsc70 monoclonal antibodies, which inhibited receptor-mediated endocytosis, blocked ligand delivery to endosomes, and caused the accumulation of ligand in clathrin-associated structures near the

cell periphery (128). Although these results support the view that Hsc70 has a role in recycling clathrin, they also suggest a role for Hsc70 prior to coated vesicle budding. Hsc70 releases clathrin, but not APs, from isolated coated vesicles (47, 165). Factors required for AP release from coated vesicles (Figure 3, *step 8*) have not been identified.

It has recently been shown that clathrin-coated vesicles isolated from brain contain a Rab-guanine nucleotide exchange factor and are able to recruit Rab5 from the cytosol (175). Rab5 is a member of the large family of small GTPases believed to control specificity in vesicle targeting, docking, and fusion events (176). Thus, among the constituents of clathrin-coated vesicles are components that ensure its accurate targeting and consumption.

MECHANISMS OF CARGO RECRUITMENT INTO COATED VESICLES

There are three classes of cell surface receptors whose entry into coated pits is controlled by sorting signals. The first, including the Tfn-R or low density lipoprotein (LDL-R), is constitutively concentrated in coated pits; the second, including EGF-R or $\beta 2$-adrenergic receptors, requires ligand-induced activation for concentration in coated pits; and the third, including CD4, is actively retained on the cell surface until its release is triggered by some signaling event (177). Other receptors, such as the insulin-R, exhibit a combination of these properties (178). Contrary to early reports (36, 179), there is no evidence that membrane proteins are excluded sterically from coated pits owing merely to the concentrated packing of selected cargo molecules there. In fact, truncated receptors lacking their internalization signals, and even inactive receptor-tyrosine kinases with their very bulky cytoplasmic domains, are passively included in budding coated vesicles at their prevailing concentration in the membrane.

Most Constitutively Internalized Receptors Carry Tyrosine-Containing Sorting Motifs

Tyrosine-containing sorting motifs remain the best defined signals for concentration of transmembrane receptors into coated pits (180). These motifs are four to six residues long and have been defined by mutagenesis studies in vivo to be both necessary and sufficient for efficient constitutive endocytosis. The two best-characterized endocytosis sorting signals are the -Tyr-Thr-Arg-Phe- motif found in Tfn-R (referred to here as the YXRF motif, because X can be any amino acid) and the -Asn-Pro-Val-Tyr- motif in LDL-R (referred to here as the NPXY motif). Initial studies on endocytosis of the LDL-R focused on the four-residue NPXY (181) motif because this sequence was found in other transmembrane receptors. However, mutation of a Phe residue two amino acids N-terminal to

the NPXY motif also severely inhibited endocytosis (176). The internalization motif in the Tfn-R (YXRF) can be functionally replaced with the tyrosine-containing internalization motifs of other proteins, but not with the four-residue internalization motif of the LDL-R (NPVY). However, the six-residue motif in the LDL-R, FDNPVY, was able to restore efficient endocytosis, although not to wild-type levels (182). These data suggest that tyrosine-containing internalization motifs are recognized by a common sorting machinery.

Based on extensive mutagenesis studies on the Tfn-R YXRF motif, the general consensus for an internalization motif is -Tyr-X–bulky-polar–hydrophobic- (180). Nuclear magnetic resonance studies on the structure of internalization motif–containing peptides in solution (183–185) have consistently revealed a propensity for forming either a β-turn or a nascent helix, despite divergence in their amino acid sequences. In a complementary approach, a search of the data base of known three-dimensional structures found that both the Tfn-R consensus (YXRF) and the LDL-R consensus (NPXY) sequences were found most commonly in other proteins within β-turns (186). This structural analysis also revealed that the aromatic side-chain of the essential Phe residue upstream from the NPXY turn in the LDL-R motif would be positioned in the same orientation as the aromatic side chain of Tyr in the Tfn-R YXRF turn (180).

AP2 molecules have been shown to interact directly with tyrosine-containing internalization sequences both on constitutively internalized receptors (such as the LDL-R or lysosomal acid phosphatase) (74, 76) and on receptors that require ligand-induced activation (such as the EGF-R) (78). Interestingly, the intracellular targeting of certain receptors to lysosomes and to the basolateral surface in polarized cells is also directed by tyrosine-containing sorting signals (180, 187). The finding (discussed above) that interaction with tyrosine-containing sorting sequences is mediated through the homologous $\mu 1$ and $\mu 2$ subunits of both AP1 and AP2, respectively (13), is consistent with this commonality in sorting signal recognition and opens up a new avenue for investigating the nature of coat protein–sorting signal interactions and their specificity.

One approach taken to unravel these complexities was to compare the ability of $\mu 1$ and $\mu 2$ to interact with different variations of a tyrosine-containing sorting motif using the yeast two-hybrid system. It was found that $\mu 2$ generally recognized these sorting motifs with higher avidity than $\mu 1$. In addition, the two μ chains were differentially sensitive to various amino acid substitutions surrounding the tyrosine and to the position of the tyrosine relative to the carboxy-terminus of the bait peptide (79). It is difficult, however, to extrapolate from data obtained with the yeast two-hybrid system to a quantitative estimate of binding affinities. Thus, a second complementary approach was to use a combinatorial peptide library (with four randomized residues on each side of a fixed Tyr-residue) to screen for peptides able to bind immobilized $\mu 2$

subunits (80). Bound peptides showed a strong consensus for the sequence Tyr-polar-Arg-Leu (although other polar and hydrophobic residues were found in positions three and four, respectively.) No other strong preference for amino acids at any other positions emerged from this analysis, indicating that there was likely no preferred structure for interaction with the isolated $\mu 2$ subunit.

The fact that the consensus sequence determined by the random library approach is essentially identical to that arrived at by mutagenesis studies of natural sorting signals in vivo argues strongly that the μ-chain interactions with target proteins provide the primary binding energy for this recognition process. This conclusion is further strengthened by the finding that the binding affinities of randomly selected peptides to immobilized $\mu 2$ chains (K_d = 1–20 μM) were comparable to those measured using intact AP2 complexes (80). However, there are several caveats to this simple yet attractive model. First, the affinities for AP2 interaction with functional internalization motifs are still relatively low ($\sim 10^{-5}$ M). Second, $\mu 2$ chain–target peptide interactions in vitro showed no structural preference (80), despite evidence for the importance of structure from in vivo studies (180, 183). Third, immobilized $\mu 2$ did not appear to interact with peptides containing the LDL-R internalization motif, NPVY, even though intact AP2 molecules bind to immobilized LDL-R cytoplasmic tails (74). Given that LDL-R and Tfn-R internalization motifs are functionally interchangeable (182), it would be expected that μ subunits would interact with peptides bearing either type of internalization motif. This apparent discrepancy may be because the full six-residue LDL-R motif, FDNPVY, necessary to function within the context of the Tfn-R cytoplasmic tail (182) was not tested. Finally, the overlapping specificities measured for recognition of tyrosine-containing sorting signals by the $\mu 1$ and $\mu 2$ chains seem inconsistent with the sorting efficiencies obtained in vivo. Thus, the possibility is still open that other subunits of the AP complex, or other components of the clathrin-coated vesicle, might participate directly or indirectly in cargo recognition and sorting. For example, conformational changes within the AP2 complex might serve to enhance or disfavor μ chain–receptor interactions. Alternatively, cooperative interactions between AP complexes might enhance avidity for certain sorting signals. Conversely, the interaction of μ chains with sorting signals may influence coat assembly or rearrangements of the assembled lattice.

Auxiliary Sorting Factors Are Required for Ligand-Induced Endocytosis

Efficient endocytosis of receptors that signal, such as receptor tyrosine kinases (RTKs) or seven-transmembrane-segment G-protein–coupled receptors (GPCRs), is triggered by ligand binding. The number of coated pits on the cell surface is not affected by activation of either the EGF-R (188) or the IgE

receptor (189). Thus, rapid ligand-induced endocytosis of this class of receptors does not require upregulation of the endocytic machinery per se but instead appears to involve activation of specific auxiliary factors that enhance the affinity and/or rate of interaction of these receptors with clathrin-coated vesicles.

LIGAND-INDUCED ENDOCYTOSIS OF RECEPTOR-TYROSINE KINASES A property that distinguishes endocytosis of RTKs from that of constitutively internalized nutrient receptors is that it is readily saturable (190, 191). Recent studies have, however, shown that very high levels of overexpression of transmembrane receptors with tyrosine-containing sorting sequences can saturate both the biosynthetic (192) and the endocytic (193) transport machinery. Saturation of RTK endocytosis does not affect endocytosis of other receptors within the same cell. These findings are important because they suggest that a distinct, readily saturable and RTK-specific component of the endocytic machinery is required for RTK internalization. Further evidence for this idea comes from the finding that recombinant insulin receptors are efficiently internalized when expressed in rat-1 cells but not when expressed in B82L mouse cells (which are able to efficiently internalize recombinant EGF-R). Moreover, fusion of insulin receptor–expressing B82L cells with nontransfected rat-1 cells restores efficient insulin-R internalization (194), suggesting that rat-1 cell cytosol can supply a factor that is absent in mouse B82L cell cytosol.

Ligand-dependent sequestration of EGF-R into coated pits has been reconstituted in vitro using perforated cells (108). EGF-R endocytosis in this assay shows similar biochemical requirements to those of Tfn-R endocytosis, and both receptors are found together in coated pits and coated vesicles after in vitro incubations in the presence of cytosol, ATP, and GTP (108). Furthermore, the requirements for endocytosis of EGF-R in vitro parallel those for endocytosis in vivo. Specifically, no ligand-dependent sequestration into coated pits is observed when cells expressing mutant EGF-R lacking either an active kinase domain or the C-terminal regulatory domain (which contains the internalization motifs) are used (109). This assay also recapitulates in two ways the requirement of EGF-R endocytosis for a limiting component of the endocytic machinery as seen in intact cells. First, ligand-dependent endocytosis of EGF-R in perforated cells requires higher concentrations of cytosol than does Tfn-R in the same cells (108). Second, cytosol from different sources differentially supports EGF-R endocytosis in vitro under conditions where Tfn-R endocytosis is unaffected. Although AP2s can form stable immunoprecipitable complexes with activated EGF-R in vivo (77, 78, 108), deletion or mutagenesis of the AP2 binding site has no effect on the rate of EGF-R endocytosis in vivo (78). This finding is consistent with in vitro results showing that soluble AP2 complexes are not rate limiting for EGF-R endocytosis (108), whereas they are

for Tfn-R endocytosis (101, 108). Taken together, these data suggest that a cytosolic component other than AP2 is required for the recruitment of activated EGF-R into coated pits.

Two candidates for an EGF-R recruitment factor have been proposed. The recent finding that Eps15, an EGF-R tyrosine kinase substrate, interacts constitutively with AP2 complexes in vivo (195) and shares sequence similarity with the *END3* gene product that causes an endocytosis defect in *Saccharomyces cerevisiae* (196) makes it an attractive candidate for such a recruitment molecule. A second report showed that microinjection of the SH2 domain of the adaptor protein grb2 inhibited endocytosis of EGF-R (197). This finding, coupled with the fact that dynamin binds to the SH3 domain of grb2 (198), led to the suggestion that grb2–EGF-R interactions were essential for endocytosis (197). However, this is unlikely because neither deletion nor point mutagenesis of the grb2 binding site on EGF-R affects its endocytosis (190). Moreover, neither the isolated SH3 domains of grb2 nor intact grb2 affect endocytosis of EGF-R in perforated cells (TE Redelmeier & SL Schmid, unpublished results). The ability to reconstitute ligand-dependent EGF-R endocytosis in vitro provides a means to identify and functionally test putative recruitment molecules.

AGONIST-INDUCED SEQUESTRATION OF G-PROTEIN–COUPLED RECEPTORS In an analogous manner to RTKs, agonist-binding to GPCRs triggers their phosphorylation (85) and their rapid internalization via a clathrin-(199, 200) and a dynamin-(115) dependent pathway. Another similarity of GPCRs to RTKs is that their internalization apparently requires an auxiliary recruitment factor. In the case of the β-adrenergic receptor (β_2AR), this recruitment factor may be β-arrestin. Phosphorylation of the β_2AR by the β-adrenergic receptor kinase (βARK) promotes binding of β-arrestin which serves to uncouple the activated receptor from the G-protein. A point mutation in the β_2AR (Y326A) is deficient both in its ability to be phosphorylated by βARK and in agonist-induced sequestration (201). The finding that the endocytosis defect can be rescued both by overexpression of βARK (201) and by overexpression of β-arrestin (202) provided evidence that β-arrestins were directly involved in β_2AR sequestration. However, others have reported that this mutation affects multiple receptor functions (203) and so the mechanism for βARK and β-arrestin rescue of the β_2AR(Y326A) mutant remains uncertain. Subsequent studies with wt β_2AR have shown that agonist binding triggers colocalization of β_2AR, β-arrestin, and clathrin in vivo and that β-arrestins bind directly to clathrin in vitro with high affinity ($K_d \sim$ 10–60 nM) (204). These results suggest that β-arrestins act as adaptor molecules to recruit GPCRs into coated pits. Since β-arrestins themselves are unable to promote clathrin assembly (J Keen, personal communication), further work is needed to determine whether β-arrestin–clathrin

interactions have any effect on subsequent AP2-clathrin interactions leading to coated vesicle formation.

Recent results from yeast have suggested that ubiquitinylation of the α-factor receptor, a GPCR, plays an essential role in its internalization (205). Interestingly, a number of RTKs have also been shown to be ubiquitinylated in mammalian cells in response to growth factor binding. However, at least for the EGF-R, this modification appears to be dependent on, rather than required for, receptor internalization (206). Thus, a role for ubiquitinylation of either RTKs or GPCRs in endocytosis in mammalian cells has not been established.

SUMMARY AND PERSPECTIVES

Since their isolation 28 years ago, clathrin-coated vesicles have been subjected to a barrage of experimental analyses that have provided detailed information about their structure and function. These analyses include subcellular fractionation, protein purification, ultrastructural examination, molecular biology, and in vitro assays for protein-protein interactions, coat assembly, membrane binding, and coated vesicle budding. The discovery that the coat constituents not only serve to shape the budding vesicle but also play a direct role in the packaging of cargo suggests that the protein sorting machinery and the vesicle budding machinery are functionally integrated. This integration leaves open the possibility that cargo molecules might themselves regulate coated vesicle formation. This interplay between coat and cargo would ensure sorting fidelity and packaging efficiency and would potentially enable modulation of vesicular trafficking in response to cargo volume and demand.

A remaining challenge is to complement our knowledge of the protein interactions that occur in solution or on the cytoplasmic surface of the membrane with an understanding of the events that occur within the lipid bilayer. Transmembrane proteins involved in coat protein docking, in cargo selection, and in vesicle budding reactions must be identified. The nature and role, if any, of localized phospholipid modifications in these events must be revealed. This additional information may be key to unraveling the mechanisms that control membrane fusion and vesicle formation.

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