REVIEW ARTICLE

Clathrin-dependent endocytosis

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The process by which clathrin-coated vesicles are produced involves interactions of multifunctional adaptor proteins with the plasma membrane, as well as with clathrin and several accessory proteins and phosphoinositides. Here we review recent findings highlighting new insights into mechanisms underlying clathrin-dependent endocytosis.

Key words: adaptor protein, AP-2, clathrin, coated pit, endocytosis, receptor.

INTRODUCTION

Eukaryotic cells exhibit at least two endocytic pathways: a clathrin-dependent pathway and a clathrin-independent pathway. The formation of endocytic clathrin-coated vesicles occurs through the interactions of cytosolic proteins with components of the inner leaflet of the plasma membrane. Clathrin-coated vesicle biogenesis is best characterized in nerve terminals, where it serves as the major pathway for recycling of synaptic-vesicle components after release of neurotransmitter in response to action potentials. This process has been used as a model for clathrin-dependent endocytosis, not least because the large number of synaptic vesicles forming at these sites facilitates investigation of the biochemical requirements for this process. Since many counterparts (isoforms or splice variants) of proteins involved in synaptic-vesicle endocytosis have been identified in non-neuronal cells, it has been suggested that the same basic machinery operates in all cells [1]. The aim of the present Review is to discuss the mechanism of clathrin-dependent endocytosis by particular focus on mammalian cells. The emphasis is on the role of clathrin and adaptor proteins in the formation of clathrin-coated vesicles. Three mechanistically defined stages, each of which corresponds to a morphologically distinct intermediate, are recognized during the formation of clathrin-coated vesicles: (1) assembly of clathrin into a polygonal lattice and formation of coated pits, (2) invagination of coated pits, and (3) pinching-off of the coated vesicles.

ASSEMBLY OF CLATHRIN AND FORMATION OF COATED PITS

Clathrin-dependent endocytosis normally occurs at specialized sites, where a complex structure, called a coated pit, is assembled in order to concentrate surface proteins for internalization. Before starting the discussion of these stages, a brief review of what is known about the main components of coated pit will be given.

THE CLATHRIN-COATED PIT IS A MULTI-COMPONENT ENDOCYTIC UNIT

The most abundant proteins found in coated pits are clathrin and the heterotetrameric protein AP-2 [adaptor (or assembly) protein-2]. Several accessory proteins are implicated in the formation of clathrin-coated vesicles, as indicated by their ability to interact with other endocytic components such as clathrin and AP-2, and by the findings that expression of fragments of these proteins that contain functional domains inhibits clathrin-dependent endocytosis. Some of these proteins are found in coated pits, but are not enriched in clathrin-coated vesicles, suggesting that they play assisting roles during clathrin-coat assembly. It is noteworthy that all of these proteins are multi-domain monomeric proteins, although homo- or hetero-oligomers are the predominant forms in which some of these proteins function. These domains are widespread and functionally specialized modules that cause the proteins to act as multifunctional molecules. In the following sections, we discuss the domain features (starting from the N-terminus and continuing to the C-terminus) of some of these proteins (Figure 1).

Clathrin

The assembly unit of clathrin, called triskelion, is a three-legged structure consisting of three heavy and three light chains. The heavy chain is an invariant polypeptide with five functionally distinct regions: the globular N-terminal domain, a relatively curved region, the 'knee', which divides the polypeptide into a distal and a proximal leg, and the C-terminal end, which mediates trimerization [1,2]. The hub region consists of three C-terminal segments of the heavy chain [3]. The N-terminal domain, which is associated with the distal leg via a flexible linker segment, is a β-propeller (based on the crystal structure of this domain) and contains a binding site (the groove between blades 1 and 2 of the β-propeller) that interacts with a number of endocytic proteins, including AP-2. The majority of clathrin-interacting proteins do so

Abbreviations used: ANTH, AP180 N-terminal homology; AP, adaptor (or assembly) protein; CALM, clathrin assembly lymphoid myeloid leukaemia; CIN85, Cbl-interacting protein of 85 kDa; Dab2, Disabled protein 2; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; EGF, epidermal growth factor; EH, Eps15 homology; ENTH, Epsin N-terminal homology; Epsin, Eps15-interacting protein; GED, GTPase effector domain; GH, growth hormone; GPCR, G-protein-coupled receptor; HIP1, huntingtin-interacting protein; JAK, Janus kinase; LDL, low-density lipoprotein; LPA, lysophosphatidic acid; PA, phosphatidic acid; PH, pleckstrin homology; PRD, proline-rich domain; PTB domain, phosphotyrosine-binding domain; RTK, receptor tyrosine kinase; SH2, Src homology 2; SH3, Src homology 3; Tf, transferrin; TGN, *trans*-Golgi network; UIM, ubiquitin-interacting motif.

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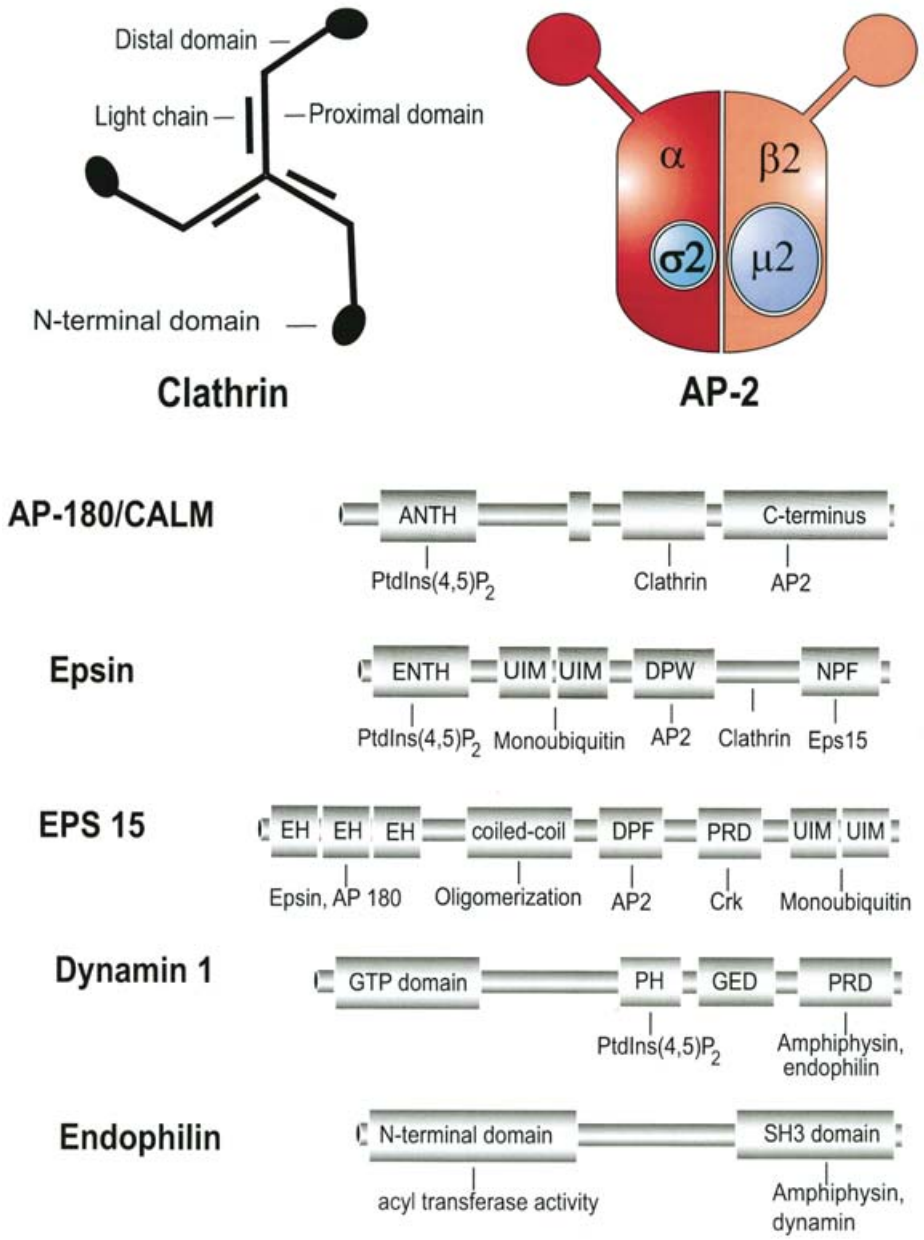

Figure 1 Some known components that play a role in the formation of clathrin-coated vesicles

Functional characteristic(s) of each domain/motif are shown. DPW, Asp-Pro-Trp; NPF, Asn-Pro-Phe; DPF, Asp-Pro-Phe; PRD, Pro-Arg-Asp. See the text for details.

through similar sequence motifs generally known as clathrin box motifs, including the LLNLD (Leu-Leu-Asn-Leu-Asp) sequence of the $\beta 2$ subunit of AP-2 [4]. Both distal and proximal legs contribute to intermolecular contacts that are necessary in order to establish closed basket structures. The proximal leg also contains binding sites for clathrin light chains [1,5,6]. There are two isoforms of the light chain (LCa and LCb), which associate randomly with clathrin heavy chains in the triskelion. The light chain has been suggested to regulate the assembly state of clathrin triskelions [7].

AP-2

AP-2 is a large protein complex composed of four subunits, denoted α, $\beta 2$, $\mu 2$ and $\delta 2$. Each of the two large (approx. 100 kDa) α and $\beta 2$ subunits consists of an N-terminal domain (called the trunk or head) and a globular C-terminal domain (called the appendage or ear), which are connected by a flexible hinge [8,9]. The α subunit appendage domain is involved in targeting AP-2 to the plasma membrane as well as in interactions with AP180, amphiphysin and endocytic proteins containing DPF (Asp-Pro-Phe) or DPW (Asp-Pro-Trp) motifs. Two binding sites on the $\beta 2$ subunit are important for the interaction of AP-2 with clathrin: a clathrin box motif in the hinge region, and a site in the ear domain [10]. $\beta 2$ may also function in the selection of specific cargo [11]. The medium-sized (50 kDa) $\mu 2$ subunit contains a phosphoinositide-binding site [12] and functions mainly in the recognition and sorting of protein cargo. Its ability to interact with $\beta 2$ may also be important for assembly of the AP-2 complex [13,14]. The small (16 kDa) $\delta 2$ subunit interacts with

the α subunit [14], but whether it has other functions is not clear.

AP180/CALM (clathrin assembly lymphoid myeloid leukaemia)

The neuronal adaptor protein AP180 (known previously as AP-3) and its ubiquitous isoform CALM are monomeric proteins with binding sites for both clathrin and AP-2 [15,16]. The N-termini of these proteins contain the ANTH (AP180 N-terminal homology) domain that mediates their binding to PtdIns(4,5)P_2-containing membranes [17]. The C-terminal region of AP180/CALM contains clathrin-binding sites with clathrin assembly activities [17]. The C-terminal region also interacts with both the α and β subunits of AP-2. *In vitro*, complex formation between AP-2 and AP180 gives a greater clathrin assembly activity than the sum of assembly activity of each protein alone, suggesting synergistic effects between AP180 and AP-2 in clathrin assembly [18].

Epsin (Eps15-interacting protein)

The N-terminal region of Epsin contains the ENTH (Epsin N-terminal homology) domain, which binds to PtdIns(4,5)P_2 and is followed by two UIMs (ubiquitin-interacting motifs) [19,20]. The central region of the protein contains a DPW domain (consisting of eight copies of the DPW motif), which mediates binding to the α subunit of AP-2, and a clathrin box motif adjacent to this domain. The C-terminal region contains an additional clathrin box followed by three NPF (Asn-Pro-Phe) motifs, which bind the EH (Eps15 homology) domains of Eps15 [21–24]. Like AP180, Epsin promotes the assembly of clathrin *in vitro*, and it has been suggested that it can interact with two clathrin terminal domains simultaneously [25].

Eps15

The N-terminal region of Eps15 [which derives its name from 'EGF (epidermal growth factor) receptor pathway substrate clone no. 15'] contains three copies of EH domains, which interact with NPF motif-containing proteins including Epsin, AP180/CALM, Numb and Esc1. The central coiled-coil region allows the protein to form homo-oligomers (dimers and tetramers) and hetero-oligomers with Eps15R and Ese1 [26]. The C-terminal region contains multiple copies of the DPF motif, which mediate binding to the α subunit of AP-2, followed by two UIMs [20,27].

Dab2 (Disabled protein 2)

Dab2 is a widely expressed protein that binds through its N-terminal PTB (phosphotyrosine-binding) domain to PtdIns(4,5)P_2 [28]. The PTB domain of Dab2 also binds to non-tyrosine-phosphorylated FXNPXY (Phe-Xaa-Asn-Pro-Xaa-Tyr) motifs found in the cytoplasmic tail of members of the LDL (low-density lipoprotein) receptor family, such as the LDL receptor, ApoER2 (apolipoprotein E receptor 2) and the scavenger receptor megalin, suggesting a role in sorting of these receptors [29,30]. The central DPF-containing region of Dab2 interacts with the appendage domain of the AP-2 α subunit [30]. The central region also contains multiple, tandemly arranged, clathrin-binding sites with clathrin assembly activity [28]. Dab2 might have a role not only in receptor sorting, but also in the general endocytic machinery, since the number of apical coated pits and vesicles is significantly reduced in Dab2 conditionally null mice [31].

Other accessory proteins

Several other molecules have been identified as components of coated pits, including HIP1 (huntingtin-interacting protein 1), hStn2 (human stonin2), intersectin/Ese and Numb [32–35] to name only a few. Other proteins that are not components of the coated pit, but are involved in later stages of clathrin-dependent endocytosis, include dynamin, amphiphysin and endophilin, which are implicated in the invagination and fission reaction.

Dynamin

Dynamin is a high-molecular-mass protein with GTPase activity. There are at least three known mammalian dynamins. Dynamin I is found predominantly in the brain, dynamin II is expressed ubiquitously and dynamin III is most abundant in testis [36]. All dynamins have an N-terminal GTPase domain involved in the binding and hydrolysis of GTP. The GTPase domain is characterized by high basal GTPase activity and low affinity for GTP. The central region has a PH (pleckstrin homology) domain, which binds specifically to PtdIns(4,5)P_2. The C-terminal region comprises a coiled-coil structure termed the GED (GTPase effector domain), which is involved in dynamin oligomerization and self-assembly. The GED is followed by a PRD (proline-rich domain), which interacts with SH3 (Src homology 3) domain-containing proteins such as amphiphysin, endophilin and actin-binding proteins [37–41].

Amphiphysin

In mammals, two forms of amphiphysin have been described. Amphiphysin 1 is expressed mainly in the brain. There are several splice variants of amphiphysin 2, some of which are present in all tissues [42]. The domain features of amphiphysin include the N-terminal coiled-coil region required for plasma-membrane interaction and formation of a heterodimer between the two forms of amphiphysin. The central region contains a domain with binding sites for clathrin and the α subunit of AP-2, followed by a proline-rich motif for endophilin binding. Finally, the C-terminal SH3 domain has been suggested to mediate interactions with dynamin and synaptojanin [42,43].

Endophilin

Endophilin has three known isoforms [endophilin 1 (SH3p4), endophilin 2 (SH3p8) and endophilin 3 (SH3p13)], whose tissue distribution patterns overlap with those of the three dynamin isoforms [44,45]. The N-terminal region of endophilin has acyltransferase activity, and its C-terminal SH3 domain interacts with dynamin and amphiphysin [44,46,47].

RELATIONSHIP BETWEEN THE FLAT LATTICE AND THE POLYGONAL BASKET

In electron micrographs, the polymerized clathrin that surrounds coated vesicles is a basket-like structure composed of pentagons and hexagons [48,49]. Geometrical requirements for a closed polygonal basket imply that 12 pentagons must always be present, but the number of hexagons may vary [49]. For example, the coated vesicles found in the brain are $\approx$76 nm in size and have 20 hexagons, whereas those from fibroblasts are $\approx$120 nm and have 60 hexagons [50]. Electron-microscopic studies have also shown that the plasma membrane of cells in culture commonly contains various amounts of polymerized clathrin in the form of flat lattices, which are composed predominantly of hexagons, and pits with varying degrees of curvature composed of both pentagons and hexagons [49]. Thus the presence of 12 pentagons appears to be necessary for the curvature of the clathrin coat, whereas the number of hexagons has to do with the size of the coat.

There are currently two views of how clathrin triskelions can assemble into the coat that surrounds the budding vesicle. The more common view suggests that clathrin triskelions first form a flat hexagonal lattice; then, in a second step, some hexagons are converted into pentagons, transforming the planar lattice into a closed sphere [6,48,49,51,52]. According to this view, flat lattices would function as precursors of coated pits. The alternative view holds that flat arrays are not precursors of coated pits, but reservoirs of clathrin available for rapid recruitment [5,53,54]. Clathrin assembly into the basket may proceed by the sequential incorporation of soluble clathrin triskelions into the growing lattice without the need for a flat hexagonal intermediate: the clathrin assembly grows so as to locate the positions of pentagons and hexagons correctly [5,53–55]. Moreover, theoretical arguments suggest that the introduction of 12 pentagons into a flat hexagonal lattice to form a spherical coat is an energetically demanding process and topologically impossible [53,54,56]. Finally, the observations that flat lattices are normally not seen at synapses [57] and that purified clathrin does not form an extended hexagonal lattice in re-assembly experiments argue against the two-step model of clathrin assembly [55]. However, several lines of evidence suggest the existence of some form of lattice remodelling. First, addressing this issue from a theoretical perspective suggests that rearrangements leading to the formation of pentagons may involve assembly and disassembly processes in which two triskelions are inserted into, or removed from, the centre of the flat lattice [58]. Moreover, energy estimates suggest that changes in clathrin lattices during rearrangement can occur by passive thermal mechanisms [59]. In addition, experiments using clathrin tagged with green fluorescent protein have indicated that exchange of free (cytosolic) clathrin for assembled clathrin on coated structures can occur even under conditions that inhibit either the invagination of coated pits or the fission of coated vesicles, suggesting rearrangements of the clathrin lattice during coated-vesicle formation [60]. Nevertheless, the question of how clathrin assembles into a closed lattice remains open.

MOLECULAR CONTACTS WITHIN THE COAT

The ability of clathrin triskelions to form both flat hexagonal lattices and closed polyhedrons of various diameters implies that some parts of the clathrin heavy chain must be flexible in order to adapt to different assemblies. The current models of a clathrin trimer suggest that the triskelion has a pucker at the vertex and that each leg has two deformable regions, the knee and the proximal leg near the vertex, which are free of contacts in the lattice. The intrinsic pucker and leg flexibility allow for modulation of the angle between legs and for a range of curvatures for a lattice when required [61]. The light chains contain a stretch of acidic residues complementary to a stretch of basic amino acids on the proximal leg of the heavy chain, which can prevent the formation of high-affinity salt bridges between anti-parallel oriented triskelions [7], suggesting that the assembly process involves electrostatic interactions between proximal legs. Furthermore, recombinant clathrin hubs (lacking the distal and terminal domains) are able to assemble into regular lattices, but cannot form closed baskets [3], indicating that the distal leg is important for the formation of complete baskets. In electron micrographs, the lattice coat of assembled clathrin is characterized by outer and inner shells of density corresponding to the hubs and distal legs and the N-terminal domains respectively. The inner shell surrounds a layer of adaptor proteins, which in turn are in contact with the vesicle membrane [62–64]. These and other observations [2] suggest that AP-2, through its interaction with the terminal domain of clathrin, acts as bridge between the lattice coat and the plasma membrane.

COATED PITS ARE FORMED AT SPECIFIC SITES ON THE PLASMA MEMBRANE

Coated pits cover some 0.5–2 % of the cell surface [65]. The formation of coated pits appears to start at specific assembly sites on the plasma membrane called ‘coated-pit zones’ [66]. Moreover, coated-pit formation occurs in a saturable manner, suggesting that the number of assembly sites is limited [67]. This is in agreement with the finding that isolated plasma membranes stripped of both clathrin and AP-2 display high-affinity binding sites for purified AP-2 [68]. Recent results also reveal that the lateral mobility of coated pits is restricted by the cortical actin network [66,69]. The membrane factors that define the assembly sites are not fully known, although some clues to the problem have been provided by recent findings. For example, phosphoinositides are generated and broken down in a regulated manner on the inner surface of the plasma membrane, and all adaptor proteins capable of promoting clathrin assembly are also able to interact with these lipids. In particular, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 have been shown to be important for both constitutive and stimulus-induced endocytosis. In addition to phosphoinositides, integral membrane proteins are required to recruit AP-2 to the plasma membrane. Candidates for providing binding sites for AP-2 are discussed in detail below.

ROLE OF AP-2 IN THE NUCLEATION OF COATED PITS

The traditional view has been that AP-2 is the adaptor protein that specifies the localization of clathrin assembly at the plasma membrane [70]. AP-2 harbours two high-affinity phosphoinositide-binding sites, one triplet lysine cluster located on the N-terminal region of the α subunit and another on the $\mu 2$ subunit, which interact independently with phosphoinositides, in particular PtdIns(4,5)P_2 [12,71]. The role of phosphoinositide binding is indicated by the finding that neomycin, a high-affinity ligand for PtdIns(4,5)P_2, and the PH domain of phospholipase Cδ, which also interacts with PtdIns(4,5)P_2, prevent the recruitment of AP-2 to membranes [12,72,73]. Furthermore, a mutant AP-2 lacking a functional phosphoinositide-binding site acts as a dominant-negative inhibitor of coated-pit formation [74].

In what sequence are clathrin and AP-2 recruited to the assembly sites? Current views suggest that coated-pit formation occurs through a two-step process in which endocytic adaptors first specify the location of clathrin assembly on the plasma membrane, and then recruit and promote clathrin polymerization. The $\beta 2$ hinge of AP-2 can interact with the N-terminal region of the clathrin heavy chain and promote lattice assembly [75]. The two-step model of clathrin assembly is supported by the following observations. Incubation in hyperosmotic medium results in the disassociation of the clathrin lattice, but leaves AP-2 complexes attached to the plasma membrane [76]. Under the same experimental conditions, HA + 8 (a chimaeric form of influenza-virus haemagglutinin capable of rapid internalization) can still interact with AP-2 [77]. Moreover, overexpression of AP180 or its clathrin-binding domain decreases the amount of clathrin associated with the plasma membrane without affecting the distribution of AP-2 [78]. These observations indicate that AP-2 can be recruited to the plasma membrane in the absence of an interaction with clathrin, and that this recruitment can occur prior to initiating clathrin assembly. Binding of a single AP-2 molecule to the plasma membrane cannot provide nucleation sites for clathrin assembly, since the β hinge/ear of AP-2 can bind only one terminal domain in the clathrin trimer [75]. AP-2 has been shown to self-associate and form clusters in solution [2,79]. Such clusters are also found in intact cells [76].

AP-2 clustering *in vivo* may, however, be mediated by tetrameric Eps15, which can interact with four AP-2 complexes [26,80], thereby creating multi-point attachment sites for the recruitment and assembly of clathrin [75,76].

ROLE OF CELL SURFACE RECEPTORS IN TARGETING OF AP-2 TO THE PLASMA MEMBRANE

Binding to PtdIns(4,5)P_2 may be only part of the mechanism controlling the targeting of AP-2 to the plasma membrane, for the following reasons. First, PtdIns(4,5)P_2, although located predominantly in the plasma membrane, is also located on the TGN (*trans*-Golgi network) (see [81]), to which AP-2 is not recruited. Secondly, the association of AP-2 with membranes is sensitive to protease treatment [68,82], suggesting that integral membrane proteins, in addition to lipids, may determine nucleation sites for clathrin assembly. One hypothesis is that surface receptors that use the coated-pit pathway for internalization may be nucleation sites for clathrin assembly [83]. In this connection, two possibilities can be distinguished. (1) Interaction with AP-2 occurs concomitantly with clathrin assembly. In this case, surface receptors may recruit AP-2 and then initiate the nucleation of coated pits; alternatively, randomly mobile receptors may collide with a flat lattice, become incorporated, and then serve as nucleation sites for further lattice formation [84,85]. In either case, receptor internalization is actively coupled to the formation of coated pits. (2) Interaction with AP-2 occurs subsequent to the formation of coated pits [86]. In this case, incorporation of surface receptors into coated pits occurs passively. Evidence for the idea that binding of AP-2 to receptors regulates its recruitment to the plasma membrane is the finding that expression of high levels of Tf (transferrin) receptors in mouse L cell lines increased the number of coated pits by 3–4-fold, whereas expression of a similar level of mutant receptors with a deletion in their cytoplasmic tail had no effect on coated-pit number [84]. However, although expression of high levels of Tf receptors in chick fibroblasts promotes the formation of flat clathrin lattices, the number of coated pits is not affected in these cells [85]. Moreover, the model does not explain how the specificity of AP-2 targeting to the plasma membrane is maintained, because these receptors are also present at high concentrations in uncoated vesicles and early endosomal compartments, to which AP-2 is not normally targeted [14,87].

A number of observations also indicate that targeting of AP-2 does not involve surface receptors. First, expression of high levels of endocytic receptors in cells, regardless of their endocytic signal types (see below), does not result in the increased recruitment of cytosolic AP-2 to the plasma membrane [88,89] or a change in the number/density of coated pits at the plasma membrane [89]. Secondly, AP-2 complexes that contain a mutant μ2 subunit defective in binding to surface proteins, such as the Tf receptor, are targeted correctly to the plasma membrane [90]. Thirdly, Tf uptake in cells expressing AP-2 containing mutant μ2, incapable of becoming phosphorylated at Thr-156 (due to replacement of this residue with alanine), is markedly inhibited. Nonetheless, these cells form coated pits that can incorporate AP-2 containing mutant μ2 [91]. Finally, incubation of an AP-2 preparation from pig brain in the presence of ATP, which increases the phosphorylation of the α, β2 and μ2 subunits by kinases associated with purified AP-2, enhances the affinity of AP-2 for various endocytic signals (2–4-fold) and for membranes (4-fold) to the same extent. On the other hand, treatment with alkaline phosphatase (which removes phosphate groups) decreases the affinity of AP-2 for endocytic signals by 3–15-fold, while the affinity for membranes decreases by only 2-fold. These data suggest that the affinity of AP-2 for endocytic motifs does not exactly parallel that for other membrane components. In other words, decreasing the affinity of AP-2 for receptor tails below a certain threshold does not further affect AP-2 recruitment to membranes [92].

ROLE OF SYNAPTOTAGMINS AS GENERAL DOCKING SITES FOR COATED PIT FORMATION

It has long been suggested that other surface proteins, rather than receptors, provide docking sites for AP-2 in the plasma membrane. Synaptotagmins are transmembrane proteins that contain two Ca^{2+}-binding domains, C_2A and C_2B, in their cytoplasmic region [93]. Synaptotagmin I binds with high affinity to AP-2 [82] via a dual interaction of its C_2B domain with the μ2 and α subunits. The binding site of μ2 for C_2B is distinct from that for tyrosine-based motifs [94]. An AP-2-binding property has also been described for other synaptotagmin isoforms that occur in almost all cell types ([95]; reviewed in [96]). Liposomes containing a glutathione S-transferase fusion protein containing the C_2B domain of synaptotagmin bind AP-2 and clathrin efficiently [94]. In contrast, mutant synaptotagmin I or VII lacking the C_2B domain reduces receptor-mediated endocytosis. Moreover, cells transiently transfected with truncated synaptotagmin VII exhibit a decrease in the number of cell-surface coated pits [97]. Finally, expression of synprint (a peptide that binds to C_2B and specifically inhibits the synaptotagmin–AP-2 interaction) can block receptor-mediated endocytosis [94]. Taken together, these observations argue for a functional link between synaptotagmins and the formation and function of coated pits. However, it may be that synaptotagmins co-operate with surface receptors in recruiting AP-2 [98].

ROLES OF OTHER ADAPTOR PROTEINS IN THE NUCLEATION OF COATED PITS

It has become evident that AP180 and Epsin are also involved in the initial step of clathrin assembly. The ENTH domain of Epsin and the ANTH domain of AP180 are phosphoinositide-binding sites that may function in targeting of these proteins to the plasma membrane. Moreover, the C-terminal region of AP180 and the central region of Epsin contain binding sites for both clathrin and AP-2. However, despite structural and functional similarities, the roles of these proteins in clathrin-dependent endocytosis may differ. First, these adaptors differ in their ability to promote clathrin assembly (see below). Secondly, overexpression of the C-terminus of AP180 causes a redistribution of membrane-associated clathrin; however, in cells overexpressing a mutant Epsin defective in PtdIns(4,5)P_2 binding, AP-2 is aggregated at the plasma membrane and, as a result, receptor-mediated endocytosis is inhibited, but the distribution of clathrin remains unaffected [19,24]. These data suggest that AP180 mediates recruitment of clathrin to membranes, whereas Epsin is important for the function of AP-2. This is consistent with the finding that the affinity of Epsin for the α-appendage of AP-2 is higher than that of AP180 [25]. AP180, like AP-2, is enriched in clathrin-coated vesicles (isolated from brain), but Epsin is not [21], suggesting that AP180 is a structural component of the coated pit and is required during the whole process of clathrin-coated vesicle formation, whereas Epsin is required for events preceding pinching-off of clathrin-coated vesicles and is displaced prior to this step. Consistent with this model, there is evidence that binding of Epsin to PtdIns(4,5)P_2 is not only important for its membrane targeting, but may, in addition, initiate the membrane curvature needed for the biogenesis of clathrin-coated vesicles [24]. Finally, Eps15, an interacting partner of Epsin, is located at the edges of forming coated pits, where it is in complex with AP-2 [99], but the

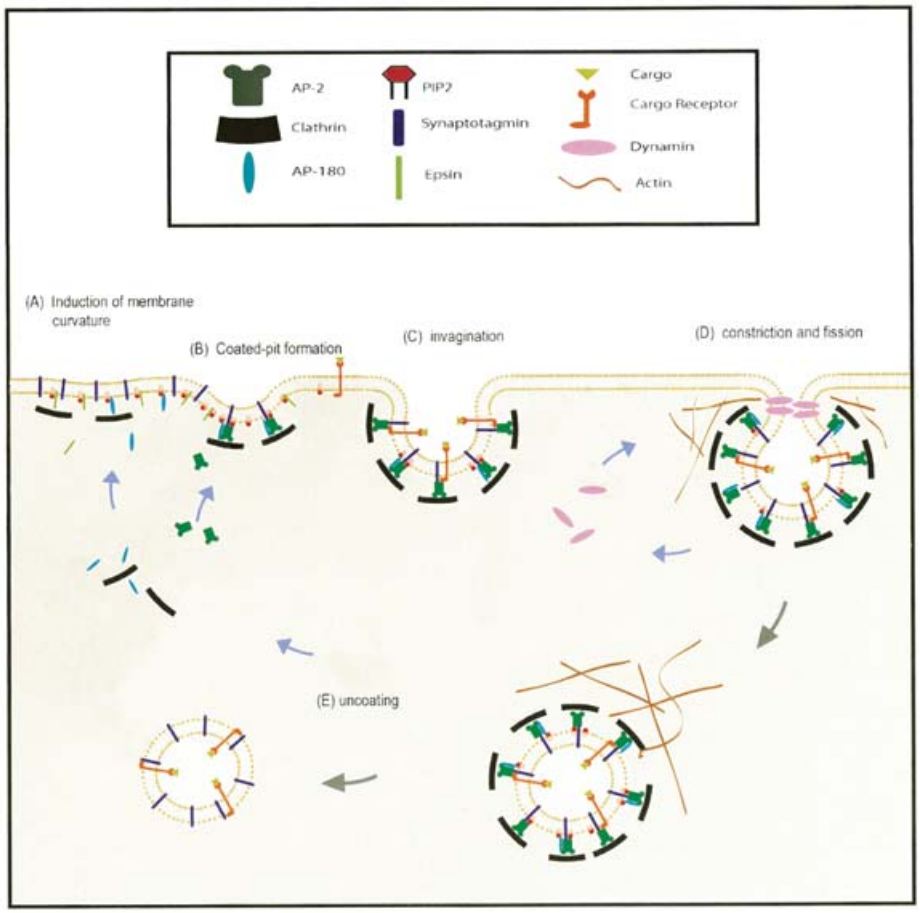

Figure 2 Model for constitutive clathrin-dependent endocytosis involving multiple steps

For clarity, only some of components involved are shown. A recently appreciated function of Epsin is the induction of positive membrane curvature upon binding to PtdIns(4,5)P_2 (PIP2)-rich sites on the inner leaflet of plasma membrane (step A). Furthermore, Epsin recruits clathrin as well as AP-2 complexes (through direct interaction or via Eps15) to the endocytic sites, where AP-2 (along with other adaptor proteins such as AP180/CALM) mediates the assembly of a clathrin cage (step B). In that way, Epsin can link membrane curvature with coated-pit formation. The shallow coated pits invaginate in a process involving proteins such as endophilin, dynamin and Rab5 (step C). The deeply invaginated coated pits pinch off from the plasma membrane in a dynamin-dependent manner (step D) to form clathrin-coated vesicles. This process may also be facilitated by endophilin and actin filaments. Constitutively recycling receptors, randomly distributed in the plane of the plasma membrane, are targeted to the forming pits via interaction with the $\mu 2$ subunit of AP-2 [196]. Modified from Trends Cell Biol., **11**, Takei, K. and Haucke, V., Clathrin-mediated endocytosis: membrane factors pull the trigger, pp. 385–391, copyright (2001), with permission from Elsevier.

Eps15–AP-2 complex becomes disrupted upon clathrin assembly [100].

The data obtained about Epsin, AP180 and AP-2 may form a basis for the following working hypothesis for coated-pit formation (Figure 2). A finite number of Epsin molecules are first recruited to the PtdIns(4,5)P_2-rich sites at the plasma membrane, where they induce membrane budding. Simultaneously with, or subsequent to, this event, Epsin and other endocytic proteins such as AP180/CALM that are now recruited to the newly formed bud facilitate the recruitment of clathrin triskelions to nucleate lattice formation. Epsin molecules then associate with AP-2 complexes by direct interaction or indirectly via Eps15, and become displaced when AP-2 molecules trigger clathrin polymerization. At this stage the edge of the forming pit is actively engaged in the recruitment of Epsin molecules (to further modulate the curvature of the pit) and the lattice grows in size, as a result of the recruitment of more AP-2 and clathrin, to encase the budding vesicle. In this scenario, the membrane-curving and AP-2-recruiting functions of Epsin are not two independent aspects, but are integrated to link the plasma-membrane curvature into AP-2-mediated cargo accumulation into budding vesicles. AP180/CALM and AP-2 have been shown to play important roles in the regulation of vesicle size [16,17,101,102]. They cannot, however, be the only determinants, because the same adaptors are operative in various cell types, and yet different cell types produce vesicles of different sizes. There is probably an additional factor(s) in each cell type that can measure the size of the vesicle and prevent it from growing further when it has reached a certain size. Note also that the formation of clathrin-coated pits and vesicles involves additional endocytic proteins, such as the clathrin-binding protein HIP1, which act together to provide the budding vesicle with a clathrin coat.

FACTORS CONTRIBUTING TO THE INVAGINATION OF COATED PITS

The fact that purified clathrin or recombinant clathrin fragments can assemble to form closed baskets indicates that the curvature of the clathrin basket is an intrinsic property of clathrin self-assembly [2]. This raises several questions. What is the relationship between the intrinsic curvature of the assembled clathrin and membrane budding? Does the clathrin coat induce membrane invagination

by providing mechanical force, or is membrane invagination a separate process, independent of clathrin assembly?

FORMATION OF SHALLOW COATED PITS

It has been suggested that the formation of the clathrin coat can provide the driving force for membrane invagination (e.g. see [58,67,103–106]). This notion is based on several observations. (1) Purified clathrin can assemble into closed basket structures with curvature similar to that of coated vesicles. (2) Planar clathrin lattices formed at 4 °C on stripped plasma membranes are transformed into deeply invaginated pits when these membranes are warmed to 37 °C [105]. (3) The size of the coats often coincides with the size of clathrin-coated vesicles [107]. (4) The energy needed to bend a clathrin net is of the same magnitude as the energy expended to bend an equivalent membrane patch [58]. Others have, however, argued that membrane invagination may, instead, be powered by forces other than clathrin assembly, and that clathrin may comply passively as the membrane curves [49,83,107,108]. One important argument against the involvement of the clathrin coat in membrane budding is that endocytosis occurs without the aid of coat proteins. In principle, several mechanisms can induce membrane invagination to form endocytic vesicles. Examples include domain-induced budding and asymmetry of surface areas. In this regard, a relevant concept is the bending energy, defined as the energy cost of bending a flat membrane [109]. It is also known that conditions that lower the bending energy can facilitate membrane budding [110].

First, studies with model membranes have suggested a simple means of vesicle formation by invagination of intramembrane domains when they have grown to a certain limiting size and become unstable. Such domain-induced budding is governed by competition between the bending energy of the domain and the edge energy of the domain [56]. Secondly, according to the 'bilayer couple model', the two leaflets of the plasma membrane may respond differently to various perturbations, causing one of the monolayers to expand relative to the other monolayer [111]. Addition of lipid to the cytoplasmic monolayer (the inner leaflet) could theoretically cause its expansion and thereby induce membrane invagination. For example, the generation of phospholipid number asymmetry between the two monolayers of the plasma membrane (by adding lipids such as phosphatidylethanolamine, which is translocated to the inner leaflet of the membrane by endogenous flippase) has been shown to provide the driving force for membrane invagination and vesicle formation [107]. Membrane invagination may also result from a change in lipid composition due to the activity of lipid-modifying enzymes. Two types of membrane lipids can be distinguished with regard to their shape. A lipid molecule with a small polar head and a large hydrocarbon chain is thought to be cone-shaped and has the ability to induce a negative membrane curvature, whereas a lipid with large polar head and small hydrocarbon chain has an inverted-cone shape, with the ability to induce positive membrane curvature. Insertion of inverted cone-like lipids into the inner leaflet of the plasma membrane induces positive curvature (bending towards the cytoplasm) of the membrane, while the presence of cone-shaped lipids induces negative curvature (bending towards the outside). The presence of inverted cone-shaped lipids in the outer leaflet has the opposite effect [112]. For example, treatment of cells with exogenous sphingomyelinase, which removes the phosphocholine headgroup from sphingomyelin, converting it into ceramide, induces the formation of ceramide-containing endocytic vesicles, which lack protein coating [113]. This effect may be due to transport of the generated ceramide to the inner leaflet via flipping, causing an increase in the surface area of this leaflet, thus inducing vesicle formation [113]. An alternative explanation could be that conversion of sphingomyelin (an inverted cone-like lipid) into ceramide (a cone-shaped lipid) in the outer leaflet will induce positive membrane curvature [112]. As discussed in the next section, local changes in lipid shape in the membrane neck induced by the enzymic activity of endophilin play an important role in the endocytic fission reaction. Epsin has been called a curvature-forming molecule because of its ability to tubulate liposomes and to promote the formation of clathrin-coated buds on PtdIns(4,5)P_2-containing monolayer membranes [24]. Both of these actions are dependent on a ligand-inducible amphipathic α-helix (named helix 0) in the N-terminal region of the ENTH domain. The outer surface of helix 0 contains hydrophobic amino acid residues, allowing membrane penetration of the helix. Although the exact mechanism of Epsin action remains uncertain, a plausible explanation is that insertion of helix 0 into the inner leaflet of the bilayer pushes lipid head groups apart, causing an increase in the surface of the leaflet, thus reducing the energy required to bend the membrane [24,114].

Cholesterol may contribute to the formation of curved pits, since, in acutely cholesterol-depleted cells, flat coated patches become more abundant than the deeply invaginated coated pits [115]. This observation has been interpreted to suggest that cholesterol depletion affects the ability of endocytic proteins, such as AP-2 and dynamin, to participate in lattice rearrangements to induce membrane invagination [115]. However, cholesterol depletion may have other functional consequences. It may, for instance, stabilize the plasma membrane (see [59]) and thereby affect the ability of endocytic adaptors to induce membrane curvature. The demonstration that cholesterol depletion affects the invagination of coated pits might also be relevant to the functions of cholesterol-binding proteins such as synaptophysin. Synaptophysin, a protein capable of self-polymerizing, has been suggested to contribute to the formation of highly curved pits [116]. It is of note that caveolin-1, which shares properties with synaptophysin, has been implicated in the formation of plasma-membrane invaginations called caveolae.

If not in membrane deformation, what then is the role of clathrin in vesicle formation? Given the need for endocytic adaptors such as Epsin to induce the initial stages of membrane budding, the role of clathrin basket is likely to be to fix and stabilize the budding membrane [24]. In further support of this model, analysis of the energetics of adaptor-mediated clathrin assembly suggests that the role of the clathrin coat is to stabilize forming vesicles, rather than providing the driving force for membrane budding [59]. Once established, the (slightly curved) pit can act as the endocytic machinery needed to facilitate the efficient concentration of selected cargo for internalization. Moreover, by recruiting motor proteins and tethering/fusion factors, coated pits can regulate the motility of endocytic carrier vesicles and their fusion with early endosomes. For instance, Rab5, a GTPase involved in the regulation of early endosomal functions and membrane trafficking [117], is recruited to forming clathrin-coated pits *in vitro* [118], although it may also play a role in the formation of deeply invaginated coated pits [119]. Nevertheless, because assessment of a possible role of the clathrin lattice in membrane budding is experimentally difficult, some contribution from the clathrin coat to this process cannot be excluded at this stage.

FORMATION OF DEEPLY INVAGINATED COATED PITS

The formation of deeply invaginated coated pits appears to be the result of the actions of several factors that act co-operatively,

leading to further alterations in the curvature of the plasma membrane. Whereas Epsin may be involved in the initial stages of membrane curvature during clathrin-coated-pit formation, endophilin and dynamin have been implicated in multiple steps during coated-vesicle formation, including the transition from the shallow stage to the deeply invaginated coated pits. The N-terminal region of endophilin has been shown to induce the tubulation of liposomes. This region also contains acyltransferase activity, but this activity is dispensable for tubulating activity [120]. A role for endophilin in the formation of deeply invaginated coated pits is compatible with the finding that the invagination of coated pits in lamprey (*Lampetra fluviatilis*) synapses is inhibited at the shallow stage by the microinjection of antibodies that interfere with endophilin functions [45]. A requirement for dynamin in the formation of deep coated pits has been demonstrated using the SH3 domain of amphiphysin (amphiphysin is needed for recruitment of dynamin to the coated pits; see below), which inhibits the formation of deeply invaginated coated pits in permeabilized cells and the ability of wild-type dynamin, but not a mutant dynamin deficient in GTPase activity, to overcome the inhibitory effect of this domain [121]. The observations that components of clathrin-coated pits, such as Dab2, can bind myosin VI (an actin-based motor protein) suggest that actin polymerization, as a force-generating factor, may also be involved in this process [122].

MEMBRANE FISSION AND THE FORMATION OF FREE COATED VESICLES

Deeply invaginated coated pits are characterized by the presence of a neck structure that connects the forming vesicle to the plasma membrane and specifies the site of fission. The neck has to be constricted sufficiently to bring the opposing membrane together in order to cause fusion within the neck and, thus, formation of a free clathrin-coated vesicle from the plasma membrane. The fission machinery includes at least three proteins. Two of these are enzymes: dynamin, with GTPase activity, and endophilin, with acyltransferase activity. The third protein, amphiphysin, functions as a linker between dynamin and clathrin coats [123]. Purified dynamin can form a tetrameric structural unit, possibly by head-to-tail interaction between two dynamin dimers, suggesting that tetramers may be the functional form of dynamin [37,124]. In low-salt solution, dynamin self-assembles into rings and stacks of rings that are interconnected as a helix [37,125]. Dynamin can also self-assemble into a helical collar at the neck of invaginated coated vesicles when nerve terminals are incubated with the slow-hydrolysable GTP analogue guanosine 5′-[γ-thio]triphosphate [126]. Self-assembly of dynamin tetramers involves interactions between the GED and the N-terminal region containing the GTPase domain [39]. Dynamin self-assembly also stimulates its GTPase activity. It has been shown that the addition of an isolated GED to dynamin tetramers results in a 50-fold stimulation of GTPase activity, whereas addition of the GED to assembled dynamin does not stimulate GTPase activity further [127]. These results suggest that the GED acts as a GTPase-activating protein for dynamin [37,127].

On the basis of these observations, one plausible model for dynamin recruitment and self-assembly may be as follows. Dynamin is recruited on to forming coated vesicles in either its GDP-bound or its nucleotide-free state, a process that is mediated by interaction of its PRD with the SH3 domain of amphiphysin. Dynamin will subsequently associate with the plasma membrane via interaction of its PH domain with PtdIns(4,5)P_2. GDP/GTP exchange then causes dynamin molecules to disassociate from the coated pits and assemble at the neck of forming coated vesicles. Upon GTP hydrolysis, the configuration of the dynamin collar will change, and concomitant with, or subsequent to, this event, GDP-bound dynamin molecules are disassembled.

Although it is widely accepted that dynamin self-assembles into a helical collar around the neck of invaginated coated pits, it is still unclear whether its assembly-stimulated GTPase activity triggers vesicle fission or whether it is used to regulate effectors that mediate the fission reaction. Two questions in this context are: (1) what conformation does assembled dynamin assume upon GTP hydrolysis, and (2) will this conformational change result in fission, or will it regulate dynamin–effector interactions? If mechano-chemical activity must be exerted to pinch off the forming vesicles, enzymes must exist that are able to convert chemical energy into mechanical forces. Dynamin has, therefore, been viewed as a mechano-chemical enzyme (or a 'pinchase') that can use the energy released from GTP hydrolysis to provide the mechanical force needed for vesicle fission [125,128]. Several models have been presented to describe the mechanical mechanism of dynamin action. An earlier study suggested that dynamin self-assembly induced by GTP binding results in the formation of a constricted coated pit, and that co-ordinated GTP hydrolysis triggers a concerted conformational change in dynamin that tightens the rings around the necks of invaginated pits such that fission can occur [125]. In support of this model is the finding that purified dynamin assembles into helical spirals on liposomes and causes their tubulation in the absence of GTP. Addition of GTP induced the formation of constricted tubules, whose outer diameter was decreased from 50 to 40 nm [129]. According to another model of dynamin action, tightening of the collar around the neck is achieved by ratcheting of one rung along the next rung of the spiral, and the energy of GTP hydrolysis is used to increase the constriction of the neck by stepwise movement of the dynamin molecules [124]. A third model suggests that the conformational change driven by the GTPase activity of dynamin is an increase in the helical pitch [130]. Using PtdIns(4,5)P_2-containing lipid nanotubes as a template for dynamin self-assembly, it was observed that dynamin in its GTP-bound state formed a tightly packed helical conformation with a pitch (corresponding to the distance between two closed rings) of 11 ± 1.5 nm. Dynamin–GDP assumed a more loosely helical conformation and extended its pitch to 20 ± 3 nm. On the basis of these observations, the authors suggested that the conformational change (i.e. stretching of the helix) driven by GTP hydrolysis provides the force needed for the fission reaction.

These mechano-chemical models predict that fission, and thereby clathrin-dependent endocytosis, depends on the assembly-stimulated rate of GTP hydrolysis by dynamin. Several studies have attempted to test these models, but they have produced conflicting results. Many dynamin variants carrying mutations in their GTPase domain (such as the T65A mutant) have been shown to inhibit fission [131], emphasizing the proposed role of dynamin as a mechano-chemical enzyme.

Other studies have, however, suggested that the main function of GTP-bound dynamin may be to recruit and activate effectors that mediate vesicle fission. Endophilin is known to interact with dynamin via its SH3 domain, and may function as a downstream effector of GTP-bound dynamin by modifying the lipid composition of the neck. It exhibits lipid transferase activity that can condense LPA (lysophosphatidic acid) and fatty acyl-CoA to PA (phosphatidic acid), and by doing so it converts an inverted-cone-shaped lipid (LPA) to a cone-shaped lipid (PA). It is reasoned that the LPA-to-PA conversion induces negative curvature in the cytoplasmic leaflet of the membrane [132]. Evidence for the involvement of endophilin in endocytosis is the finding that whereas cytosol depleted of endophilin did not

support vesicle formation, addition of recombinant endophilin restored this process. Moreover, a mutant of endophilin lacking the SH3 domain but retaining its acyltransferase activity was not able to support coated-vesicle formation.

It has been estimated that an increase in the helical pitch distance (as a result of stretching of the collar) may cause the neck to collapse and may drive the fission reaction on its own. On the other hand, although the decrease in the inner radius of the dynamin helix caused by tightening of the collar is insufficient to drive fission, it can, nevertheless, be a contributory factor. Moreover, estimation of the amount of LPA that has to be converted into PA in order to provide negative curvature in the cytoplasmic monolayer of the neck also shows that the endophilin-mediated LPA-to-PA conversion can alone drive the fission reaction [112]. It is therefore conceivable that dynamin and endophilin act jointly to induce fission. In this regard, the enzymic activity of endophilin may be concentrated on the isolated part of membrane between two rings of dynamin ([112]; see also [133]). The regulatory action of dynamin may not, however, be restricted to regulation of the enzyme activity of endophilin. It has long been appreciated that the actin cytoskeleton, in addition to its possible roles in restricting the sites of coated-pit formation in the plasma membrane and in the invagination of coated pits (see above), also plays a role in late events (at the constriction and/or fission steps of endocytosis) of the formation of coated vesicles in some cell types [134–136]. In support of this, dynamin 1 and 2 have been found to interact with a variety of actin-binding proteins containing an SH3 domain, including profilin, mammalian Abp1 (actin-binding protein 1) and cortactin [40,41,137]. Arp2/3 (actin-related protein 2/3) is a protein complex that promotes the nucleation of actin filaments, and cortactin has been shown to stimulate its nucleation-promoting activity [138]. Moreover, dynamin 2, through its interaction with cortactin, can regulate actin polymerization [139]. Finally, the GTPase domain of dynamin has been shown to be important for its role in actin filament assembly [140]. Thus dynamin, acting as a GTPase, is likely to have a direct role in the regulation of actin filament assembly at sites of endocytosis, which can then provide force to induce the scission of forming vesicles. The recent observation that dynamin and actin are recruited sequentially to endocytic sites [141] also supports the hypothesis that dynamin may act as the upstream factor that regulates actin polymerization at the site of vesicle formation.

COUPLING OF COATED PIT FORMATION TO CARGO UPTAKE

Surface proteins can enter coated pits accidentally by lateral diffusion in the lipid bilayer and then be captured specifically by coated-pit components. Alternatively, entry of cargo proteins into coated pits may be mediated by targeting proteins that mediate selective transport to the coated pits. Receptor-mediated endocytosis can be constitutive or triggered by signals. The sorting activity of the constitutive pathway is based on the recognition of a linear peptide in the receptor tails, while the signal-induced pathway employs a range of adaptors and covalent modifications to facilitate receptor internalization.

CONSTITUTIVE PATHWAY OF ENDOCYTIC UPTAKE

The constitutive pathway is involved mainly in the uptake of receptors that undergo continuous internalization and recycling, but it may also account for the ligand-independent internalization of signalling receptors [142,143]. The biological role of the constitutive pathway comprises processes such as the uptake of macromolecules and viruses from the circulation and regulation of the number of proteins at the surface. The receptors involved are referred to as ‘transport’ or ‘nutrient’ receptors to emphasize their functions. It is generally assumed that internalization of constitutively internalizing receptors is similar in the absence and presence of ligand [70,144,145]. However, a number of observations indicate that this rule is not without exceptions. At least in the case of the asialoglycoprotein and Tf receptors, internalization of the receptor seems to be stimulated by the ligand [146,147]. Transport receptors may also have signal-transduction functions. Early studies on receptor uptake suggested that cytoplasmic domains of endocytosing proteins contain targeting information that allow them to be concentrated in the coated pits, as mutations in these domains inhibit receptor endocytosis [148,149]. It is now known that the cytoplasmic tails of many surface receptors contain motifs that are recognized by components of the coated pits. Other non-receptor proteins that undergo endocytosis, such as TGN38, a TGN-resident protein, also contain such signals. The most common endocytic signals are the tyrosine-based and leucine-based motifs. Two types of tyrosine-based motifs are known: NPXY (Asn-Pro-Xaa-Tyr), as found in the members of the LDL-receptor family, and YXXØ (Tyr-Xaa-Xaa-Øaa, where Ø/Øaa is an amino acid with a large hydrophobic side chain such as leucine, isoleucine or methionine) motifs, such as YTRF (Tyr-Tyr-Arg-Phe) in the Tf receptor [150] and YQRL (Tyr-Gln-Arg-Leu) in TGN38 [151]. Leucine-based motifs consist of two successive leucine residues, but in some cases there is a large hydrophobic residue (such as isoleucine or methionine) instead of leucine. Several leucine-based motifs have been implicated in the internalization of surface proteins, including the Leu-Leu pair in the C-terminal region of GLUT4 [152], and the Leu-Ile and Met-Leu pairs in the tail of the invariant chain [153]. The cytoplasmic domain of CD4 also contains a dileucine (Leu-413, Leu-414) motif, which is essential for both the constitutive and stimulus-induced endocytosis of CD4 [154]. It should also be noted that phenylalanine is a conserved substitution for tyrosine in tyrosine-based motifs such as FAAV (Phe-Ala-Ala-Val) found in the asialoglycoprotein receptor [155]. Moreover, it is likely that some of these endocytic motifs are parts of larger endocytic sequences, as is the case for leucine-based motifs in the tail of invariant chain and YGVF (Tyr-Gly-Val-Phe) in P-selectin [156,157]. In contrast, Tyr-388 appears to be the major determinant that mediates internalization of the lysosome-associated membrane protein Lamp1 [158]. It is also of note that the PTB domain of Dab2 binds the six-residue tyrosine-based motif FXNPXY, rather than NPXY [29,30]. Matters become more complicated, as the cytoplasmic tails of several proteins contain multiple potential signals. For example, the LDL receptor contains a typical tyrosine-based motif (YXXØ) in addition to its FXNPXY motif, and megalin has three copies of the FXNPXY motif [29,159,160].

The importance of internalization signals in regulating the endocytic activity of surface proteins is evident from mutation studies, which have shown that substitution or deletion of individual or combined residues decreases the internalization efficiency of surface proteins to various extents, and from studies with chimaeric transmembrane proteins containing a cytoplasmic domain with an internalization signal that induces the internalization of proteins that are normally excluded from coated pits. Recognition and binding of endocytic signals in the cytoplasmic tails of surface proteins to be incorporated in coated pits is mediated by adaptor proteins. With regard to the recruitment of proteins containing tyrosine-based motifs, an important role has been attributed to the $\mu 2$ subunit of AP-2, which can interact specifically with tyrosine-based motifs of the Tf receptor, TGN38 and several other surface proteins [90,151,157]. Crystal

structures of the motif-binding domain of $\mu 2$ in the presence of peptides containing tyrosine-based motifs have revealed that these motifs are bound in an extended β-strand conformation, and recognition appears to be based on hydrophobic interactions [106]. The functional role of the $\mu 2$ subunit for the internalization of constitutive receptors is supported further by the observation that the endocytosis of Tf receptors in cells expressing mutant $\mu 2$ subunit is several-fold lower than that in control cells [90]. The mechanism by which leucine-based motifs are recognized is not fully known. Leucine-based motifs have been shown to interact with the $\beta 2$ subunit of AP-2, although relatively weakly, which may explain the low rate of internalization of proteins containing leucine-based motifs [11,161]. Interactions between the first leucine-based (Leu-Ile) motif from the invariant chain and the $\mu 2$ subunit have also been reported [162].

The diversity of endocytic motifs raises the question as to whether the endocytic sorting machinery involves adaptor proteins other than (or in addition to) AP-2. It is now established that activated signalling receptors recruit proteins with properties of adaptor molecules that function in regulating receptor internalization. These adaptor proteins serve as 'connectors' or 'docking proteins' [163,164] that link the activated receptor to components of coated pits, such as AP-2 (see below). The endocytosis of some constitutively internalizing receptors may also depend on such adaptor proteins. For example, Dab2 interacts with the FXNPXY motif of megalin (a member of the LDL-receptor family involved in the re-uptake of several plasma proteins in kidney) through its PTB domain, and it has been implicated in the sorting of megalin and probably other FXNPXY-bearing receptors into coated pits [29,122]. In support of this role, overexpression of a Myc–PTB $\times$ 2 of Dab2 (i.e. two PTB domains in tandem) inhibits the internalization of LDL receptors without affecting the uptake of Tf receptors [28].

SIGNAL-INDUCED PATHWAY OF ENDOCYTIC UPTAKE

Signalling receptors are surface proteins that undergo increased internalization upon ligand stimulation. A major function of this pathway of internalization is to control the signalling potency of the receptor by regulating events that occur at the level of internalization, which in turn determine the post-endocytic fate (recycling, retention or degradation) of the receptor. Mechanisms of ligand-induced internalization are not defined, but various data suggest that they are distinct from the mechanism(s) mediating constitutive internalization. For instance, ligand-induced phosphorylation of the receptor and components of the coated pits that act as connectors plays an important regulatory role in receptor signalling and uptake. Moreover, it is now evident that mono-ubiquitination of components such Eps15 and Epsin can regulate their endocytic functions, and there is also some evidence that mammalian RTKs (receptor tyrosine kinases) may utilize mono-ubiquitin as a signal for endocytosis. Ubiquitination consists of conjugation of a single molecule or chains of ubiquitin, a 76-amino-acid polypeptide, to the target proteins in a process involving three enzymic components: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase). Two structurally distinct families of E3 have been identified. The Hect (*h*omologous to *E*6-AP *C*-*t*erminus) domain-containing E3s such as Nedd4 contain substrate-binding domains and a Hect domain, which forms a thioester bond with ubiquitin and then transfers it to a lysine residue of the substrate. The RING (*r*eally *i*nteresting *n*ew *g*ene)-finger containing E3s, such as c-Cbl and Mdm2, contains E2-binding and substrate-binding domains, facilitating the transfer of ubiquitin from E2 to the target protein [165]. E3s may also be binding partners for proteins with a role in endocytosis; thus, in addition to conferring specificity to the system, they can act as endocytic adaptors. Here we will discuss recent data on mechanisms involved in the internalization of three classes of signalling receptors by focusing on some prototype receptors from each class.

RTKs

RTKs such as the EGF receptor possess intrinsic protein tyrosine kinase activity. Ligand binding causes a change in the conformation of the monomeric receptor that leads to the non-covalent dimerization of two receptors [166]. Dimerization facilitates tyrosine autophosphorylation, and the phosphotyrosines in turn provide docking sites for downstream effectors containing SH2 or PTB domains. One popular hypothesis proposes that a ligand-induced conformational change in the receptor tail would expose the masked endocytic motifs (see below), enabling the receptor to interact with AP-2 and thereby to be recruited into coated pits. However, results from several studies indicate that this mechanism is not sufficient for ligand-stimulated internalization of the receptor. For example, internalization of the EGF receptor, despite it containing a tyrosine-based (YRAL; Tyr-Arg-Ala-Leu) and three NPXY motifs [167], does not seem to be affected by mutations that prevent binding to AP-2 [168]. Moreover, cells expressing a mutant AP-2 $\mu 2$ subunit exhibit normal internalization of the EGF receptor, whereas Tf receptor uptake is blocked in these cells [90]. Finally, in experiments with perforated cells, sequestration of the Tf receptor has been found to increase with an increase in the amount of AP-2, whereas sequestration of the EGF receptor is unaffected at any AP-2 concentration, suggesting that AP-2 is not limiting for EGF-receptor internalization [169]. Ligand-induced internalization of the EGF receptor has been shown to depend on intrinsic tyrosine kinase activity, but receptor autophosphorylation is not required for endocytic activity, as mutant receptors lacking any autophosphorylation sites can undergo ligand-induced internalization [170]. These observation suggest that tyrosine phosphorylation of the endocytic connector(s) is necessary for the regulation of receptor internalization.

Eps15 has been identified as an essential component of the EGF-receptor endocytic pathway, as accelerated internalization of activated receptors has been shown to be dependent on tyrosine phosphorylation of Eps15 [171]. It has been suggested that interaction of Eps15 with the activated EGF receptor may be mediated by the adaptor protein c-Crk, as the oncogenic form of this protein (v-Crk) can bind directly to the activated EGF receptor, possibly via binding of its SH2 domain to the phosphorylated YLIP (Tyr-Leu-Ile-Pro) motif. The SH3 domain of v-Crc binds Eps15, which in turn mediates association of the activated receptor with AP-2. Evidence in favour of this model is the observation that a mutant EGF receptor unable to bind Crk neither binds nor phosphorylates Eps15 [172]. Other data suggest that the binding of dynamin to the EGF receptor through the adaptor protein Grb2 is required for receptor internalization, as indicated by microinjection experiments showing that a Grb2 mutant defective in dynamin binding inhibits EGF receptor uptake [173].

Although ligand-induced ubiquitination of mammalian surface receptors and its role in the sorting of internalized receptors to the lysosomes has been known for some time [174], it is still unclear whether receptor ubiquitination itself is required for plasma-membrane internalization. Mono-ubiquitination of the receptor tail may create binding sites for endocytic adaptors that

possess UIMs. Data consistent with this hypothesis come from a recent study showing that the mono-ubiquitination of several UIM-containing endocytic proteins, including Eps15 and Epsin, occurs concomitantly with activation of the EGF receptor [175]. In particular, Nedd4-mediated mono-ubiquitination of Eps15 following the activation of the EGF receptor strongly suggests that this modification may regulate the ubiquitin-binding ability of its UIM, which in turn may recognize the ubiquitinated receptor, providing a potential means by which the activated receptor is recruited into coated pits [175].

c-Cbl, which mediates the ligand-induced ubiquitination of several RTKs, has also been implicated in the regulation of receptor internalization For instance, $Cbl^{-/-}$ macrophages exhibit reduced uptake of the colony-stimulating-factor-1 receptor [176]. Recent studies indicate that, following recruitment to the activated EGF receptor, c-Cbl becomes tyrosine-phosphorylated and then acts as a bridge between the receptor and the complex between CIN85 (Cbl-interacting protein of 85 kDa) and endophilin [177]. CIN85 is known to interact with a variety of proteins, including tyrosine-phosphorylated c-Cbl, via interaction of its N-terminal SH3 domains and PRDs. In addition, the C-terminal PRD of CIN85 mediates constitutive binding to the SH3 domain of endophilin. Expression of a mutant CIN85 that retains the ability to bind endophilin but is unable to form a complex with Cbl delays receptor degradation. Moreover, expression of a mutant CIN85 that is able to bind c-Cbl but fails to bind endophilin has the same effect [177]. A study of the role of Cbl in the regulation of hepatocyte-growth-factor-receptor endocytosis has further shown that expression of a mutant endophilin, lacking enzymic activity but still able to bind CIN85, impaired ligand-induced internalization and degradation of this receptor [178].

Although it may be only part of the story, c-Cbl-mediated recruitment of the CIN85–endophilin complex to the activated receptor before internalization may be meaningful, providing a high local enzymic activity of endophilin and thereby affecting invagination/fission processes. These data suggest that the ubiquitinated components of coated pits, such as Eps15, function during the sorting step, facilitating receptor recruitment into coated pits, whereas adaptor proteins such as Grb2 and Cbl function during later stages (invagination/fission) of clathrin-coated-vesicle formation by facilitating the generation of receptor–Grb2–dynamin and receptor–Cbl–CIN85–endophilin complexes respectively. c-Cbl is also found in association with several other RTKs [179], and it remains to see how general this mechanism could be.

CYTOKINE RECEPTORS

Members of the cytokine receptor family, including the GH (growth hormone) receptor, lack intrinsic kinase activity, but utilize members of the JAK (Janus kinase) family to exert their effects [180]. Cytokine receptors undergo dimerization in the presence of bivalent ligands such as GH [181]. The ligand-induced dimerization of GH receptors promotes the kinase activity of receptor-associated JAK2 enzymes, which initiate cross-phosphorylation of themselves as well as of the associated receptors Phosphorylated JAK2 mediates many of the intracellular actions of the receptor through interaction with signalling molecules such as STAT (signal transducers and activators of transcription). However, JAK2-mediated tyrosine phosphorylation of the receptor does not seem to be necessary for efficient GH-dependent receptor internalization. Thus a mutant GH receptor that fails to bind JAK2 and consequently cannot be phosphorylated following ligand binding is internalized at the same rate as the functional receptor [182]. Ligand-induced internalization of the GH receptor requires functional ubiquitin machinery, as uptake and degradation of the receptor in cells with a temperature-sensitive defect in ubiquitination is considerably reduced [183]. Ubiquitination and ligand-induced internalization of the receptor have been shown to depend on a 10-amino-acid motif (the UbE motif) in the receptor tail, as both processes are inhibited in cells expressing a GH receptor mutant in which the UbE motif is inactivated. However, a GH receptor mutant unable to become ubiquitinated can still be internalized [184,185], suggesting that receptor ubiquitination itself is not a necessary link to the internalization process. It seems likely that the UbE domain acts as the binding site for recruitment of E3, which regulates the functions of proteins that cause receptor internalization by facilitating their ubiquitination [184]. Alternatively, the ligase activity of E3 may not be important, and it may instead have an adaptor role in linking the receptor to components of coated pits.

GPCRs (G-PROTEIN-COUPLED RECEPTORS)

The main structural feature shared by all GPCRs is seven helical transmembrane domains connected by alternating intracellular and extracellular loops [186]. GPCR activation and endocytosis occur through a complicated process involving heterotrimeric G-proteins, which mediate many of the actions of the ligand, as well as β-arrestins (β-arrestin 1 and 2) that link activated receptors to the clathrin endocytic pathway. It has been shown for several GPCRs that these receptors are also able to form homo- and hetero-dimers. Although ligand binding may play a role in stabilizing pre-formed dimers, evidence supporting a general role for ligands in inducing dimerization of GPCRs is lacking. Emerging data, however, suggest that constitutive or ligand-induced heterodimerization of some GPCRs may result in a new receptor with altered signalling and trafficking properties [187]. The picture becomes more complex when considering (1) the different mechanisms by which receptor responsiveness to the agonist is decreased, and (2) different preferences of GPCRs for β-arrestins and the impact of these events on receptor signalling and trafficking.

ACTIVATION OF GPCRs AND THE ROLE OF β-ARRESTINS IN RECEPTOR DESENSITIZATION

According to models based on allosteric mechanisms, GPCRs can intrinsically have several conformational states, which exist in some equilibrium in the absence of ligand (see [186] for a review). Ligands that behave as agonists are able to stabilize the active state of the receptor, which acts as a guanine nucleotide exchange factor for heterotrimeric G-proteins. G-proteins are composed of an α subunit and a stable $\beta\gamma$ dimer, which form a trimeric complex when the α subunit is in its GDP-bound state. Upon GDP/GTP exchange on the α subunit (which has intrinsic GTPase activity), the latter dissociates from the receptor as well as from the $G\beta\gamma$ complex. There are several types of α subunits, and different GPCRs work through distinct G-proteins. Depending on the type of subunit, the GTPase activity of α subunits can mediate activation or inhibition of downstream effectors. For example, $G\alpha_s$ subunits activate adenylate cyclase, whereas $G\alpha_i$ subunits inhibit this pathway. On the other hand, $G\beta\gamma$ liberated from $G\alpha$ subunits regulates effectors such as GPCR kinases, which in turn phosphorylate, among others, the C-terminal tails of agonist-occupied receptors on serine/threonine residues (reviewed in [188]). Following GTP hydrolysis, the GDP-bound α subunit

re-associates with the G$\beta\gamma$ complex and can again become activated by the receptor. There are several mechanisms that can terminate signalling via G-proteins. One such mechanism involves binding of β-arrestin to the agonist-occupied GPCRs following phosphorylation of the receptors by GPCR kinases. This uncouples the receptor from the G-protein, preventing it from further signal transduction to G-proteins. This process is referred to as 'homologous desensitization' [189]. Moreover, as discussed below, the binding of β-arrestins is required for the targeting of many, although not necessarily all, GPCRs to the clathrin-coated pathway of endocytosis.

ROLE OF β-ARRESTINS IN GPCR INTERNALIZATION (SEQUESTRATION)

The endocytic function of β-arrestins is evident from their ability to interact with GPCRs, AP-2 and clathrin. Interaction with phosphoinositides and recruitment of ubiquitin ligases as well as signalling molecules further suggest how β-arrestin-dependent endocytosis might be regulated. GPCRs are classified into two classes based on their affinities for β-arrestins. Class A receptors, such as β_2-adrenergic, μ-opioid and dopamine DIA receptors, bind preferentially to β-arrestin 2 and form low-affinity transient complexes, which dissociate upon internalization of the receptor. In contrast, class B receptors, such as the angiotensin II type 1A and vasopressin 2 receptors, possess similar affinities for both β-arrestin proteins and form high-affinity stable complexes leading to internalization of the receptor–β-arrestin complex [190,191]. β-Arrestins can interact with the β subunit of AP-2 through their C-terminal domains [193]. The interaction between β-arrestin and AP-2 has also been substantiated by experiments in which fluorescently tagged β_2-adaptin was redistributed from the cytoplasm to the β_2-adrenergic receptor–β-arrestin complex at the plasma membrane following agonist treatment [164]. β-Arrestins can also bind clathrin via a region upstream of the β_2-adaptin binding site, but they are not able to promote clathrin lattice assembly [164,192]. Expression of C-terminal constructs of β-arrestin 2 lacking either the clathrin or the AP-2 binding sites results in inhibition of β_2-adrenergic receptor internalization, suggesting that interactions of β-arrestin with both clathrin and AP-2 are important for receptor endocytosis. However, whereas a β-arrestin 2 mutant lacking the clathrin binding site is recruited to coated pits, β-arrestin 2 lacking the AP-2 binding site is not [193]. These observations suggest that interaction of β-arrestin with AP-2, rather than clathrin, is the essential targeting step recruiting GPCRs to coated pits, whereas the subsequent β-arrestin–clathrin interaction contributes to receptor internalization. A β-arrestin 2 construct deficient in binding to PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 does not localize to clathrin-coated pits and fails to support effective receptor endocytosis [194], suggesting that interactions of β-arrestin with both constitutively produced and signal-induced phosphoinositides also contribute to the incorporation of activated GPCRs into clathrin-coated pits. It has been shown that Mdm2-mediated ubiquitination of β-arrestin 2 is necessary for β_2-adrenergic receptor internalization, but ubiquitination of the receptor by a second, as yet unidentified, ubiquitin ligase mediates receptor down-regulation. β-Arrestin 2 binds and recruits both ubiquitin ligases responsible for these ubiquitinations [195]. How β-arrestin ubiquitination affects receptor internalization is currently unknown.

SUMMARY

The identification and elucidation of the roles of adaptor proteins in clathrin-dependent endocytosis has advanced our understanding of the molecular mechanisms by which this process occurs. AP-2 is the adaptor that seems to be involved in nearly all stages of clathrin-coated-vesicle formation. The recognition of the role of other endocytic adaptors and lipids in this process indicates, however, that the formation of coated pits involves a complex series of protein–protein and protein–lipid interactions. In contrast with the earlier suggestion that clathrin assembly functions to induce membrane curvature, it now seems that it is used as a stabilizing coat for the components of the sorting machinery that concentrates plasma-membrane proteins into the clathrin-coated vesicles. While constitutively recycling receptors bearing tyrosine-based or leucine-based endocytic motifs may associate with AP-2 (by direct and indirect links) to internalize, the uptake of signalling receptors cannot easily be explained in terms of conventional endocytic signals. Phosphorylation of adaptor proteins as well as of the receptor itself seems to be important for the uptake of the signalling receptors. The fact that some of the components of clathrin-coated pits have ubiquitin-binding domains suggests that they might function in the sorting of mono-ubiquitinated receptors into the clathrin-dependent endocytic pathway. Future functional studies as well as theoretical analysis will, hopefully, provide a more complete understanding of the dynamic interplay of components of the coated pit.

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