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Dynamics of Endocytic Vesicle Creation

Review

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Summary

Clathrin-mediated endocytosis is the main path for receptor internalization in metazoans and is essential for controlling cell integrity and signaling. It is driven by a large array of protein and lipid interactions that have been deciphered mainly by biochemical and genetic means. To place these interactions into context, and ultimately build a fully operative model of endocytosis at the molecular level, it is necessary to know the kinetic details of the role of each protein in this process. In this review, we describe the recent efforts made, by using live cell imaging, to define clear steps in the formation of endocytic vesicles and to observe the recruitment of key proteins during membrane invagination, the scission of a newly formed vesicle, and its movement away from the plasma membrane.

In their seminal paper of 1964, Roth and Porter described how numerous "coated pits" could be visualized by using thin-section electron microscopy (EM) at the plasma membrane of oocytes of the mosquito *Aedes aegypti* after a blood meal (Roth and Porter, 1964). Based on simple observations coupled with careful measurement and interpretation, they proposed that coated pits represent membrane invaginations that pinch off the plasma membrane to form coated vesicles, and that, after internalization, coated vesicles uncoat and deliver their cargo of protein to an intracellular target; in this case, the developing yolk. Although the components of the coat were then unknown, this study delineated the essential features of clathrin-mediated endocytosis (CME), one of the most important routes of receptor internalization in eukaryotic cells. Subsequent studies, conducted by using a combination of biochemistry, EM, and genetics, demonstrated that the coat is made up of a lattice of the protein clathrin (Pearse, 1976), that clathrin-coated pits (CCPs) are ubiquitous in eukaryotic cells, and that a consortium of proteins work together to drive the formation and invagination of CCPs and catalyze the scission and uncoating of clathrin-coated vesicles (CCVs) (reviewed in Brodsky et al., 2001; Mousavi et al., 2004; Slepnev and De Camilli, 2000). Later work showed the importance of CME in somatic cells for nutrient uptake, for efficient signaling from some types of receptors (Cavalli et al., 2001), and for efficient receptor downregulation and signal attenuation (Polo et al., 2004), and it established the central role played by CME in synaptic vesicle recycling in neurons (Morgan et al., 2002).

Perhaps not surprisingly, given its central role in receptor internalization, CME has been implicated in numerous disease states, including cancer (Floyd and De Camilli, 1998; Polo et al., 2004), several congenital disorders of the central nervous system (Arai et al., 2002; Cataldo et al., 2000; Velier et al., 1998), and viral infection (Sieczkarski and Whittaker, 2002). Thus, given the importance of CME in cell homeostasis and disease, considerable effort has been expended in understanding how the CME machinery works, and in placing this in the wider context of cell signaling at the plasma membrane. In this review, we will discuss current models of CME, and we emphasize the growing importance of live-cell imaging studies in the pursuit of a fuller understanding of this essential process.

The Classical Model of CME

The very earliest studies of CME suggested that this was a dynamic mechanism, but measuring and quantifying these dynamics was made difficult through a reliance on static EM images to detect "snapshots" of intermediate stages in the formation of a CCV (Higgins and McMahon, 2002). CCPs were imaged in a variety of cell types in various stages of invagination by using both thin-section EM and, in fibroblasts adhering to a coverslip, deep-etch EM (Heuser, 1980; Miller et al., 1991). Then, by ordering a gallery of images into a temporal sequence, a smooth progression from small, partially invaginated CCPs through to large, deeply invaginated CCPs was suggested and integrated into a basic model of CME (Heuser, 1980). The duration of the entire process was estimated indirectly by comparing the proportion of plasma membrane occupied by CCPs, about 2% (Goldstein et al., 1979; Griffiths et al., 1989), with the measured rate of plasma membrane uptake through CME, which is 1%–5% per minute (Bretscher, 1984; Steinman et al., 1983). Therefore, if one CCP gave rise to one CCV, CCP lifetime was simply the ratio of these two numbers, which was 20 s to 2 min.

Subsequent investigations of CME focused on the proteins and lipids that drive CCP invagination and CCV budding, and several routes have typically been explored to establish the role(s) played by different components. First, candidate endocytic proteins were identified as major components of purified CCVs (Pearse, 1976), or their homologs were discovered through genetic screens for endocytic mutants in yeast (reviewed in D'Hondt et al., 2000) or *Drosophila* (Chen et al., 1991; Kosaka and Ikeda, 1983; van der Bliek and Meyerowitz, 1991). Yet, other proteins and lipids were identified biochemically as binding partners of proteins with pre-

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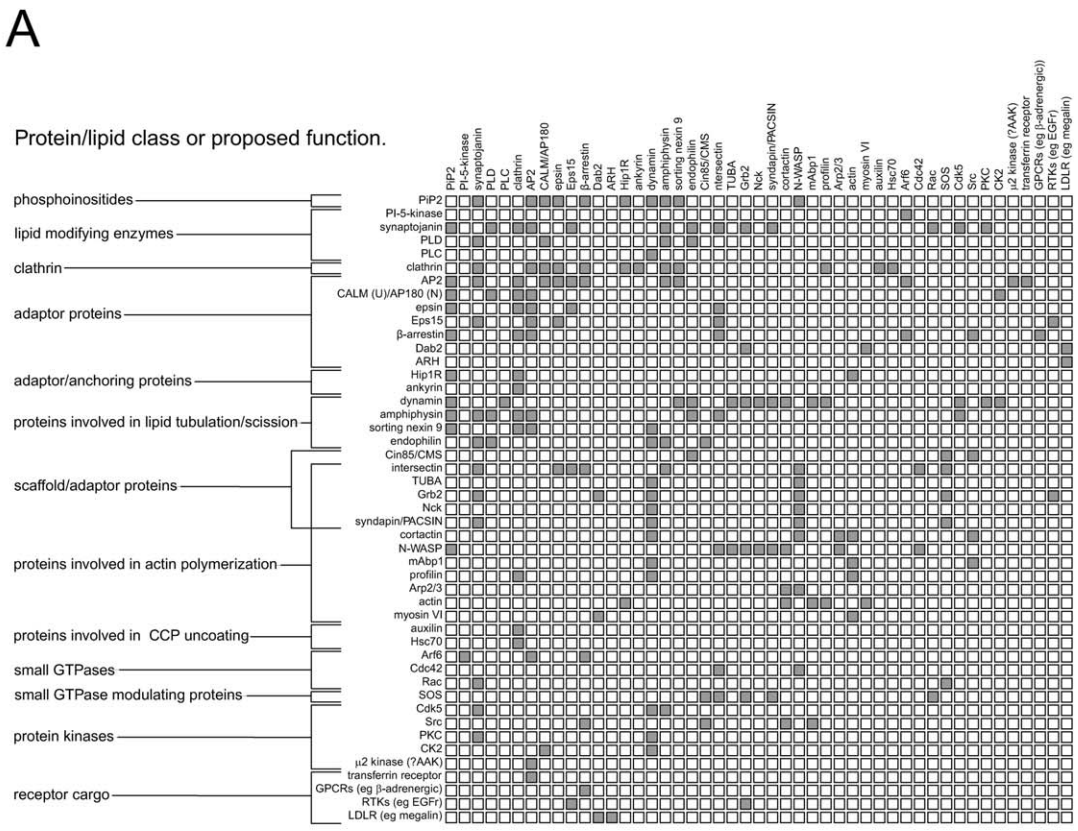
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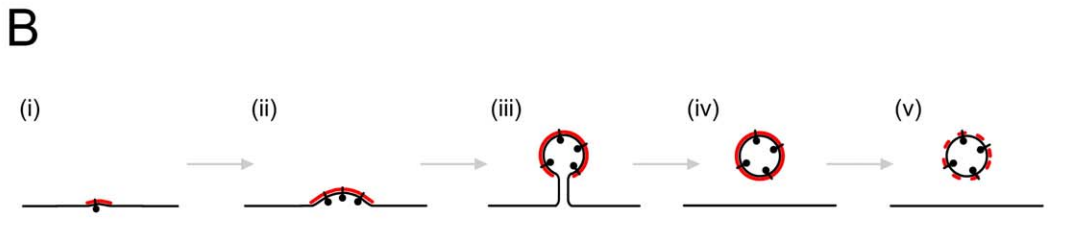
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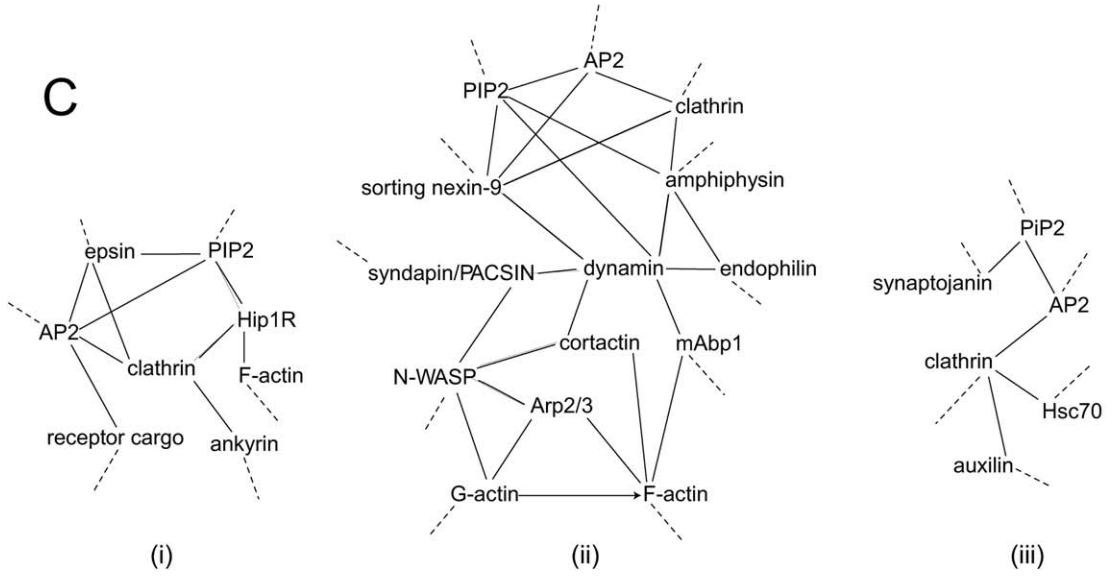
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viously established roles in CME (Benmerah et al., 1995; McPherson et al., 1996; Ringstad et al., 1997). Second, based on the domain structure and biochemical properties of the candidate protein, tools were developed to specifically interfere with the protein function, and their effect on the efficiency of ligand uptake via CME was assessed. By correlating the effects of targeted intervention with the accumulation of various stages of CCP invagination during CCV formation in intact (Damke et al., 1994; Shupliakov et al., 1997) or semipermeabilized cells (Smythe et al., 1992), the role played by different proteins and lipids in the different stages of CME was inferred. Third, complementary work with minimal systems in vitro explored the biochemistry of specific steps in detail, including the early stages of clathrin recruitment and CCP growth (Ford et al., 2002), membrane tubulation (Takei et al., 1998), and scission (Stowell et al., 1999).

Collectively, decades of such work has been integrated into the "classical" model of CME (Figure 1). At the heart of this model lies a molecular machine, held together and controlled by a convoluted network of protein/protein and protein/lipid interactions (Figure 1A), that drives the morphological changes in CCP geometry described by EM studies (Figure 1B). At each stage in coated pit invagination and budding, it is thought that different sets of protein/protein and protein/lipid interactions are brought into play in a precisely orchestrated series of submechanisms (Figure 1C). In a simple version of this model, CME begins when a clathrin coat is nucleated at a site on the plasma membrane through recruitment by the adaptor protein 2 complex (AP-2). The clathrin coat grows at its edges through polymerization of clathrin to form a coated pit, where receptors destined for internalization concentrate through interaction with adaptor proteins. In a series of geometric changes driven by protein/lipid interaction (Farsad and De Camilli, 2003), the CCP invaginates, forming a thin membrane neck, which is severed through the action of the large GTPase dynamin in combination with accessory proteins (see Hinshaw, 2000 for a review). After scission, the receptor-laden CCV uncoats and delivers its receptor cargo to the endosomal system.

One Model Fits All?

As with many models of complex biological phenomena, the classical model of CME requires caveats and modifications for it to be applied to specific cellular contexts. For instance, this model holds well in synapses where the largest CCPs are of a similar size to CCVs (30–50 nm diameter), and one CCV apparently forms from one CCP (Heuser and Reese, 1973). However, in other cell types, such as fibroblasts, the situation is less clear. Here, as well as CCPs of a similar size to fibroblast CCVs (100–150 nm diameter), larger CCPs (>300 nm diameter) have been imaged with multiple domains of curvature or relatively flat areas linked to deeply invaginated portions (Heuser and Anderson, 1989; Miller et al., 1991; Rappoport et al., 2004), suggesting that vesicles can also form from only part of a large lattice. However, because it was difficult to explain how the invagination of only part of a large, flat clathrin lattice could be energetically favorable, it was suggested that they were "dead end" or redundant structures, and not true precursors of CCVs (Kirchhausen, 2000).

In addition to CCP size and topology, the properties of CME also differ between cell types. For instance, CME in neuronal terminals is Ca^{2+} dependent (Gad et al., 1998), and, unlike fibroblast CME (Lamaze et al., 1997; Merrifield et al., 2005; Yarar et al., 2005), it is not sensitive to perturbation of the actin cytoskeleton (Sankaranarayanan et al., 2003; but see Shupliakov et al., 2002). Indeed, even within the same cell, CME can show remarkably different properties within different domains of the plasma membrane. Thus, in polarized epithelial cells, CME at the apical surface is sensitive to drugs that perturb actin polymerization, while CME in the basolateral membrane is not (Gottlieb et al., 1993).

Such cell-specific differences in CME are reflected in the consortium of endocytic proteins expressed by different cells. Some of the protein components involved in CME, such as clathrin, dynamin, and AP-2 appear to be part of a core mechanism common to all cell types, while others appear to be cell-specific accessory proteins that give rise to the varied properties of CME observed (Mousavi et al., 2004). However,

Figure 1. An Overview of the Protein Network Involved in CME

(A) Interactions among some of the proteins and lipids implicated in CME in different cell types (note that this is not a comprehensive list, and numerous proteins have been omitted due to space constraints). Each gray square represents a putative interaction between components based on biochemical data including yeast two hybrid, coimmunoprecipitation, gel overlay, or assays designed to probe protein/lipid binding. For the sake of simplicity, not all protein isoforms are shown, and the tissue specificity of expression has not been indicated.

(B) Geometric changes in the classical model of endocytosis. (i) A CCP (red) nucleates on the plasma membrane and recruits receptors (lollipops) through adaptor proteins. (ii and iii) The CCP then grows and invaginates to form a deeply invaginated CCP. (iv and v) Scission liberates a CCV (iv) that rapidly uncoats (v).

(C) Some of the interactions and mechanisms thought to be important during different stages of the classical model of CME. Solid lines indicate established interactions; broken lines indicate that other binding partners are known, but they have not been included for the sake of simplicity. (i) Nucleation of a CCP is thought to start when AP-2 binds to PIP2 at the plasma membrane and recruits clathrin and receptors. The nascent CCP is anchored to the submembrane cytoskeleton via Hip1R, and CCP growth and invagination occur through a process involving epsin. (ii) The formation of a deeply invaginated CCP and scission is driven by a consortium of proteins, including dynamin, amphiphysin, and endophilin, which aid the constriction of CCPs and formation of a thin membrane neck. Dynamin forms a collar around the membrane neck and drives scission via GTP hydrolysis. The trigger for dynamin GTP hydrolysis may include the interaction of several different components, including PIP2, sorting nexin-9, amphiphysin, and dynamin itself. Dynamin also has established links with the actin polymerization machinery via syndapin/PACSIN, profilin (not shown), cortactin, and mAbp1. (iii) Uncoating of a completed CCV involves a consortium of proteins, including the PI phosphatase synaptojanin, which is thought to hydrolyze PIP2 at newly formed CCVs, thus causing AP-2 and clathrin to dissociate from the CCV surface. Dissolution of the coat is aided by auxilin and Hsc70.

drawing the line between core and accessory proteins has not always been trivial, and this problem has been compounded by uncertainty surrounding the precise function of some proteins essential for CME, such as the large GTPase dynamin. Thus, although dynamin clearly plays a pivotal role in scission during CME in a wide variety of cell types, it is not clear whether dynamin acts exclusively as a mechanoenzyme, a classical GTPase, a sensor, or some combination of all of these (Sever et al., 2000). Moreover, dynamin has clear links with proteins involved in actin polymerization, prompting the suggestion that dynamin and actin work together during CME (Qualmann and Kessels, 2002). However, the role of actin in CME is ambiguous (Fujimoto et al., 2000), and so it is unclear whether the links between dynamin and proteins involved in actin polymerization represent a characteristic of the core mechanism of CME, or whether they represent cell-specific characteristics.

Many of these issues could be clarified if it was possible to image a high-resolution, time-resolved series of clearly identified intermediates in the formation of a CCV in a given cell type, and to know precisely which proteins and lipids were present at each step and which interactions were important at each time point for the progression of the entire structure to the next stage in the moments leading up to scission. One way of approaching this ideal has been to use EM to capture intermediates during CCP invagination and scission, and this strategy may be developed further by using EM tomography (Higgins and McMahon, 2002). An alternative approach, with lower spatial, but excellent temporal, resolution, is to use live-cell fluorescence imaging (Rappoport et al., 2004).

Imaging Clathrin Dynamics in Living Cells

In 1999, Gaidarov et al. reported the first use of green fluorescent protein (GFP) fused to the N terminus of clathrin light chain a (LCa) to follow the dynamic behavior of CCPs in live cells by using fluorescence microscopy (Gaidarov et al., 1999). It was shown that GFP-LCa bound to clathrin heavy chain incorporated into coated pits and did not perturb the normal uptake of transferrin. Subsequent work showed that LCa could also be labeled with DsRed and other fluorescent proteins, and that these probes could be used to label CCPs without interfering with CME (Engqvist-Goldstein et al., 2001; Merrifield et al., 2002; Rappoport and Simon, 2003).

Time-resolved fluorescence images provided, for the first time, a glimpse of the life of individual CCPs, and these observations had clear correlates with the classical model of CME. GFP-LCa fluorescence on the plasma membrane was clustered in clathrin-coated structures (CCSs), either in diffraction-limited spots, likely corresponding to single CCPs, or larger structures, likely corresponding to aggregates of CCPs or flat clathrin lattices. The distribution of CCS sizes varied among different cell lines and was further described in subsequent studies (Ehrlich et al., 2004; Merrifield et al., 2002, 2005; Rappoport and Simon, 2003). Moreover, Gaidarov et al. showed that CCSs formed at the plasma membrane and grew in intensity (nucleation and growth) before suddenly disappearing (movement and uncoating), over a total period of 20–80 s, similar to previous indirect estimates of CCP lifetime. During their time at the plasma membrane, the majority of CCSs remained relatively static or showed short-range, random motion as if constrained by the submembrane cytoskeleton (Gaidarov et al., 1999). This population most probably corresponded to individual CCPs based on size, colocalization with CCP markers such as AP-2 (Gaidarov et al., 1999; Merrifield et al., 2002), and their relative immobility (Engqvist-Goldstein et al., 2001; Gaidarov et al., 1999; Merrifield et al., 2002). A small proportion of CCSs was seen to undergo longer-range, directed motion along microtubules (Rappoport et al., 2003b), and it was later shown that CCP markers such as AP-2 were absent from these motile structures (Keyel et al., 2004; Rappoport et al., 2003a). It was thus suggested that this class of CCSs corresponded to endosomal organelles. Confidence that the more commonly observed CCS dynamics were related to CME was strengthened by the shared sensitivity of CCS dynamics and CME to a range of interventions. For example, CCP movement was shown to be sensitive to high-osmolarity stress (Keyel et al., 2004), a procedure known to block CME and CCP formation (Heuser and Anderson, 1989). Also, blocking actin polymerization by using the G-actin-sequestering drug latrunculin blocked CCS dynamics in neuronal dendrites (Blanpied et al., 2002) and in fibroblasts (Merrifield et al., 2005; Yarar et al., 2005). Since the block in CCS dynamics on latrunculin treatment coincided with a block in transferrin uptake, it was suggested that the observed CCS dynamics were related to endocytosis (Blanpied et al., 2002; Yarar et al., 2005).

However, although CCS dynamics observed in live cells undoubtedly corresponded to CME, some debate arose regarding specific observations made in different studies. In an early study, many CCSs seemed to form at specific sites on the plasma membrane, and multiple clathrin punctae (presumably CCVs) were occasionally seen to emerge from discrete sites and travel over short distances before disappearing (Gaidarov et al., 1999). This prompted the suggestion that “hot spots” of CCV formation exist, representing specific sites at the membrane at which CCP formation can repeatedly nucleate (Gaidarov et al., 1999). Consistent with these observations, in cultured neurons, CCPs were observed specifically at dendrites around synapses and formed repeatedly at the same locations, suggesting that hot spots also exist in these cells and correspond to specific structures around postsynaptic densities (Blanpied et al., 2002). In contrast to the targeted nucleation of CCPs at specific sites, later work with confocal microscopy in epithelial cell lines suggested that CCVs do not repeatedly form at the same site, but that, instead, CCPs are nucleated at random over the cell surface (Ehrlich et al., 2004). Moreover, CCSs of low intensity were usually short lived (<30 s) and were thought to correspond to CCPs failing to produce a CCV. Consequently, only CCPs stabilized by recruitment of receptors would successfully mature to form a CCV, and thus CME could only be mediated by CCPs that recently formed de novo (Ehrlich et al., 2004). However, several lines of evidence suggest that this is probably not the

only way in which CCPs operate. First, it was shown that activated G protein-coupled receptors (GPCR) are recruited to preexisting CCPs at the cell surface and do not require de novo formation (and stabilization) of CCPs for internalization (Santini et al., 2002; Scott et al., 2002). Similar results have been shown for activated epidermal growth factor receptors (EGFR) (Benesch et al., 2005; and our unpublished data). Second, in mature (3–4 weeks), cultured neurons, the CCSs observed at the plasma membrane appeared stable, and yet the cells were still able to internalize transferrin (Blanpied et al., 2002). Thus, de novo formation and disappearance of CCSs, as measured by using fluorescence microscopy, is not a prerequisite for endocytic activity, and apparently "stable" CCSs can still mediate endocytosis. This is consistent with fluorescence recovery after photobleaching (FRAP) experiments showing that stable CCSs at the cell surface still undergo active clathrin exchange with the cytosol (Moskowitz et al., 2003; Wu et al., 2001), which may be partly due to CCV formation under permissive conditions. Finally, mobile clathrin punctae were occasionally seen to separate from static CCSs (Blanpied et al., 2002; Gaidarov et al., 1999; Merrifield et al., 2005; Rappoport et al., 2003a; Yarar et al., 2005), prompting the suggestion that mobile CCVs can bud off of larger CCSs, which remained as static structures at the cell surface and regrew after CCV formation (Rappoport et al., 2003a). Hence, it was suggested that these large CCSs might correspond to the large CCPs with multiple budding domains seen in fibroblasts by using EM (Rappoport et al., 2004).

Collectively, these observations suggest that two types of CCSs observed by using fluorescence microscopy were potentially involved in CME. The first population corresponded to individual CCPs that grew de novo, matured, and formed single CCVs, while the second population consisted of a heterogeneous population of larger CCSs from which CCVs could bud.

Refining the Criteria for CCV Formation

From these early studies, it became apparent that using fluorescence microscopy to precisely define when a CCV was born during CME in mammalian cells was a major problem. One obvious marker for scission and liberation of an individual CCV was a marked increase in lateral mobility of a CCS as an anchored CCP transformed into an untethered CCV, followed shortly by disappearance as the CCV uncoated (Ehrlich et al., 2004; Gaidarov et al., 1999). However, precisely when invagination and scission occurred during such sequences remained unclear due to the limited spatial resolution of the imaging techniques used. In an effort to circumvent these resolution constraints, evanescent field (EF) microscopy was used to monitor clathrin dynamics during CME (Benesch et al., 2005; Keyel et al., 2004; Merrifield et al., 2002; Rappoport and Simon, 2003; Yarar et al., 2005). EF microscopy uses total internal reflection of excitation light at the glass/water interface at the surface of a coverslip (and hence the other popular name of the technique, TIRF microscopy) to form an evanescent field, a thin layer of light that decays exponentially in intensity with distance in the z axis from the surface of the coverslip into the overlaying buffer (Steyer and Almers, 2001). Consequently, EF microscopy provides similar resolution to thin-section EM in the z axis and can be used to detect changes in proximity of fluorescently labeled organelles relative to the plasma membrane with submicron resolution as they dim (move away from the glass/water interface) or increase in fluorescence (move closer to the glass/water interface). In living cells, it is necessary to control for changes in organelle fluorescence due to the loss or gain of fluorophores, and, for structures close to the plasma membrane, this is achieved most simply by using EF and wide-field epifluorescence (Epi) illumination in tandem (Merrifield et al., 2002, 2004; Yarar et al., 2005). Using this approach, it was shown that CCPs moved into the cell during CME (Merrifield et al., 2002; Yarar et al., 2005), and that dynamin was recruited to CCPs shortly before they moved away from the plasma membrane, while recruitment of N-WASP, Arp2/3, and actin peaked later (Merrifield et al., 2002, 2004) (Figure 2). In fibroblasts, the discovery that dynamin was recruited to internalizing CCSs before actin was consistent with a putative role for actin polymerization downstream of dynamin during CME (Qualmann and Kessels, 2002).

In parallel with this study, the relationship between CCS dynamics and the dynamics of accessory proteins labeled with GFP was analyzed by different groups in a variety of different cell types by using Epi, confocal, or EF fluorescence microscopy. Several broad conclusions may be drawn from the published data, and endocytic proteins can be divided into three groups based on their dynamic relationship to CCSs.

For the first group of proteins, the relative fluorescence of the accessory protein closely matches fluorescence changes of clathrin at sites of CCP nucleation, growth, and disappearance. This class includes cell surface receptors that are constitutively endocytosed, such as the transferrin receptor (Tfnr), labeled directly or with bound transferrin (Ehrlich et al., 2004; Merrifield et al., 2005; Rappoport et al., 2005), and proteins involved in the basic organization of CCPs and their anchoring to the plasma membrane, such as AP-2 (Ehrlich et al., 2004; Keyel et al., 2004). It should be noted, however, that more careful examination of the relationship between AP-2 and CCSs has revealed that AP-2 is excluded from internalizing CCSs (Rappoport et al., 2003a), and that it is excluded from smaller CCSs as they bud off of larger CCSs (Rappoport et al., 2005). Therefore, the apparently stoichiometric relationships between clathrin and adaptor proteins may be dramatically modulated at CCSs during the CME cycle, though the mechanistic significance of these observations is not yet clear. Another protein that showed similar dynamics to CCSs was huntingtin interactin protein 1R (Hip1R), which binds to clathrin and F-actin and has been implicated in anchoring CCPs to the submembrane actin cytoskeleton (Engqvist-Goldstein et al., 2001). Hip1R is apparently required for productive association of CCSs and the actin plumes generated during CCS internalization, since knockdown of Hip1R expression by using siRNA led to a reduction of CME efficiency and to the stable association of dynamin, cortactin, and actin with CCPs at the cell surface (Engqvist-Goldstein et al., 2004).

The second group of proteins is comprised of those

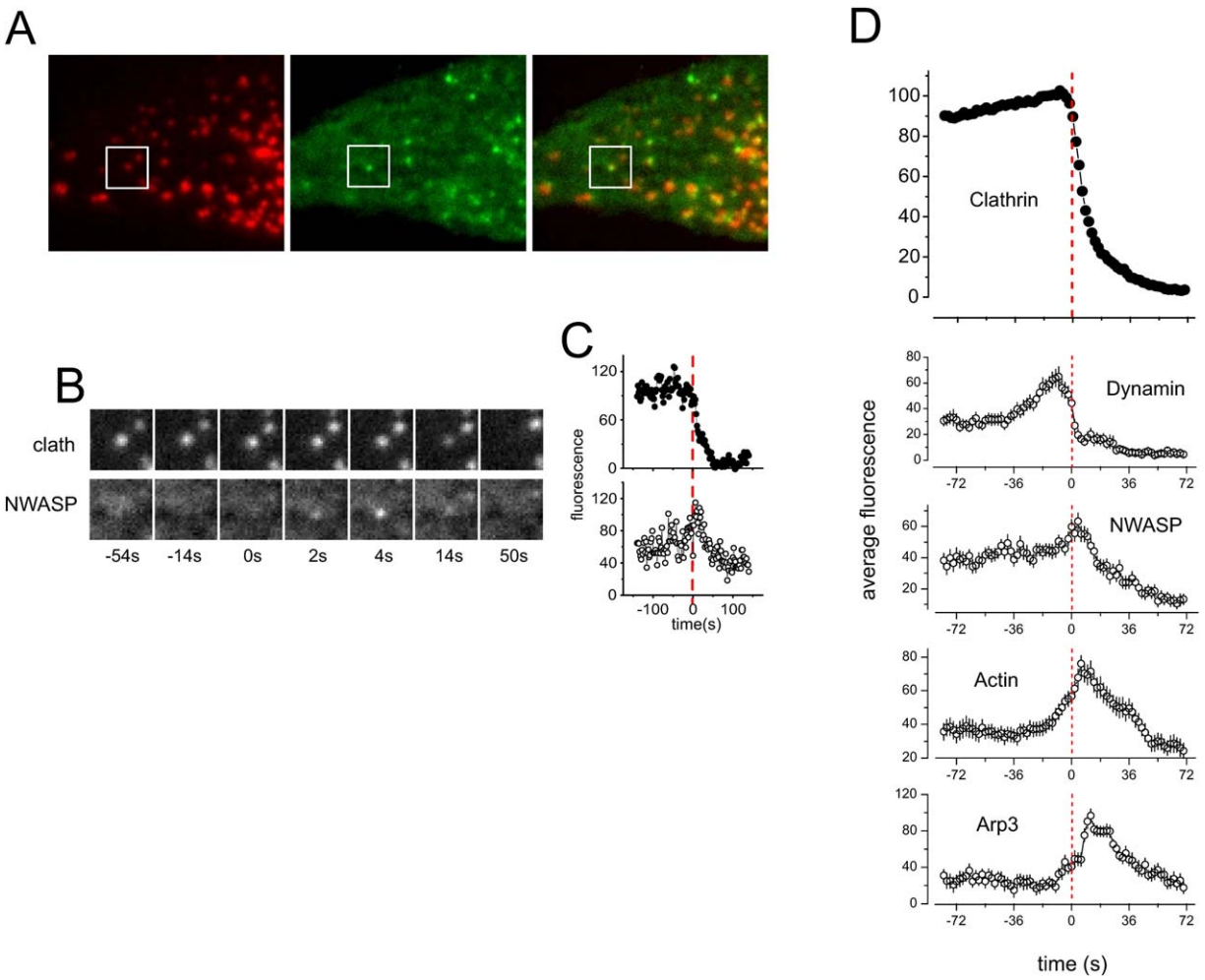

Figure 2. Endocytic Proteins Are Recruited to Internalizing CCPs from a Cytosolic Pool

(A) Part of a Swiss 3T3 cell imaged by using EF microscopy to reveal CCSs labeled with clathrin-DsRed (left panel), N-WASP labeled with GFP (middle panel), and overlaid (right panel).

(B) Time-resolved series of images showing a single internalizing CCP. The CCP (upper panel) dims as it moves away from the plasma membrane and out of the illuminating evanescent field. N-WASP was transiently recruited from a cytosolic pool. The recruitment of N-WASP was presumably triggered by a coordinated web of protein/protein and protein/lipid interactions. Dynamin is recruited to CCPs just prior to internalization, while N-WASP, actin, and Arp3 are recruited later. The averages are ± SEM.

(C) The fluorescence changes associated with this endocytic event were quantified, and a time origin corresponding to the initiation of CCP internalization was defined.

(D) Using this analysis strategy, it was shown that endocytic proteins show different recruitment "profiles" relative to CCP internalization.

proteins that are transiently recruited to CCPs from a cytosolic pool (Figure 2). This rapidly expanding group includes dynamin (Merrifield et al., 2002; Rappoport and Simon, 2003), a large GTPase essential for CME (Hinshaw, 2000) whose late recruitment presages CCP internalization in fibroblasts (Figure 2). Other proteins that are transiently recruited from a cytosolic pool include sorting nexin-9 (Soulet et al., 2005), which binds to and stimulates the GTPase activity of dynamin (Lundmark and Carlsson, 2003; Soulet et al., 2005); Arp3 (Merrifield et al., 2004), a component of the Arp2/3 complex necessary for dendritic nucleation of actin meshworks (Pollard and Beltzner, 2002); neural wiskott aldrich syndrome protein (N-WASP) (Benesch et al., 2005; Merrifield et al., 2004), an activator of the Arp2/3 complex (Miki and Takenawa, 2003); WASP interactin protein (WIP) (Benesch et al., 2005), an activator of N-WASP (Higgs and Pollard, 2001); cortactin (Merrifield et al., 2005), a binding partner of dynamin; N-WASP, an activator of the Arp2/3 complex (Daly, 2004); actin itself (Benesch et al., 2005; Merrifield et al., 2002; Yarar et al., 2005); and, finally, Nck (Benesch et al., 2005), an SH3 adaptor protein (Buday et al., 2002). It should be noted that some of these proteins, like dynamin or N-WASP, are present also at earlier stages at the CCP, though in lower amounts (Figure 2D, see also [Ehrlich et al., 2004; Rappoport and Simon, 2003]), and could thus have roles at several stages of CCV creation.

The third group of proteins is comprised of those proteins that are only recruited to CCPs on activation of specific receptors. This group includes signaling receptors themselves, such as GPCRs and EGFRs, which are

recruited to preexisting CCPs upon receptor ligand binding and activation ([Benesch et al., 2005; Scott et al., 2002], also our unpublished data), and the adaptor proteins involved in receptor recruitment. The latter group includes β-arrestin, which is recruited to preexisting CCPs upon activation of GPCRs (Santini et al., 2002; Scott et al., 2002), and the adaptor proteins Grb-2 and Sos, which are apparently recruited to CCPs upon activation of EGFRs (Benesch et al., 2005).

For any given accessory protein that showed a distinctly different pattern of dynamics to CCPs, either constitutively or in response to a specific signal, there was presumably a set of signals that triggered the recruitment and dismissal of those proteins. These signals must have involved the extensive protein/protein and protein/lipid interactions described between endocytic proteins (Figure 1) under the control of a network of kinases and phosphatases (Pelkmans et al., 2005). Thus, using the dynamics of accessory proteins as a read-out, it is now possible to directly access the signals controlling protein recruitment to clathrin-coated structures, and to monitor the resulting dynamics of these structures during receptor-mediated endocytosis. The power of this approach is best exemplified by an elegant series of studies with null mutants and live cell imaging of tagged proteins to investigate the dynamics of receptor-mediated endocytosis in yeast. It has been known for some time that receptor-mediated endocytosis in yeast is actin-dependent, and a large body of evidence indicates that the sites of endocytosis are defined by "actin patches" at the cell cortex. However, the role played by clathrin in yeast receptor-mediated endocytosis has been ambiguous (Baggett and Wendland, 2001), and so the similarity between yeast and metazoan receptor-mediated endocytosis has remained unclear (Munn, 2001).

Two recent studies by Kaksonen et al. have demonstrated that the yeast endocytic system may be even more similar to clathrin-mediated endocytosis in mammalian cells than previously thought (Kaksonen et al., 2003, 2005; reviewed in Merrifield, 2004). Because yeast cells are spherical, endocytic events can be effectively viewed from the side by using Epi microscopy focused at the "equator" of the cell. By fitting the microscope point spread function to quasipunctate (<150 nm) fluorescent objects, the small inward movement made by actin patches could be correlated with the recruitment of fluorescently labeled endocytic proteins (Kaksonen et al., 2003). By assessing the dynamics of the actin patch markers Sla1-GFP, Pan1-GFP, or Abp1-GFP in different deletion mutants, the relative contribution of different protein components to actin patch dynamics could be assessed through comparison of these markers in wild-type cells. Moreover, the investigation of patch dynamics in deletion mutants led to the discovery of some strikingly aberrant actin patch dynamics.

In wild-type cells, the inward movement of actin patches was found to occur in two stages, under the control of a number of proteins grouped in at least four "modules" according to their appearance at the actin patch. First, the presumptive site of actin patch formation was marked by the formation of a clathrin patch (Kaksonen et al., 2005). As the patch matured, additional components of the "coat module" were recruited that included Sla1p (an adaptor protein), Pan1p (a yeast homolog of Ede1p), Sla2p (a homolog of Hip1R), and others. This coat complex formed independently of actin, but its subsequent movement and disassembly required actin polymerization. Next, a phase of actin polymerization and slow movement over ~200 nm was presaged by the recruitment to the cell cortex of a module of Arp2/3 activators including the WASP family member Las17p and the myosin 1 family member Myo5p. Surprisingly, the Arp2/3 activators remained as a static spot at the cell cortex as an actin plume developed, consistent with the idea that the plume grows "upside-down" with the barbed ends abutting the plasma membrane and the pointed ends addressing the invaginating bud. The developing actin plume was marked by recruitment of an "actin module" comprising actin, Sac6p (a homolog of fimbrin), Cap1p/2p (actin capping proteins), Abp1p (an activator of the Arp2/3 complex), and members of the Arp2/3 complex. Finally, an "amphiphysin module" consisting of Rvs161p and Rvs165p (homologs of amphiphysin) was recruited after actin polymerization had started, an event that may have marked vesicle scission. Rapid inward movement was associated with the dissipation of the actin plume.

Both this and another study (Newpher et al., 2005) show a clear link between clathrin and actin patches in yeast, a link that has hitherto remained elusive. Surprisingly, it also shown that in the absence of clathrin, actin patches could still form, albeit with much reduced efficiency. Once formed, these patches could mature into vesicles with only slightly altered kinetics, showing the nonobligatory role of clathrin in yeast receptor-mediated endocytosis, as predicted previously (Baggett and Wendland, 2001). Moreover, the general pattern of clathrin patch movement and actin polymerization shows striking similarities to the results described previously in metazoan cells (Figure 2). However, in these studies in both yeast and mammalian cells, despite the clear correlation between clathrin patch internalization and recruitment of endocytic proteins, the precise moment at which scission occurred and a completed vesicle was liberated remained obscure (Santini and Keen, 2002). In the case of mammalian cells, this prompted a debate as to whether actin polymerization and the formation of actin plumes might be involved in boosting completed vesicles away from the plasma membrane, or whether it might be involved in force generation during a late stage of invagination and scission (Merrifield, 2004; Qualmann et al., 2000; Santini and Keen, 2002). To address this problem, the precise relationship between the dynamics of clathrin-coated structures and the time of vesicle creation required further clarification. It was thus necessary to devise an optical assay to pinpoint the moment of coated vesicle formation.

Detecting Scission at Single CCPs

How does one detect the conversion of an individual clathrin-coated pit into a clathrin-coated vesicle? Early studies used EM to probe the accessibility of coated pits to external membrane impermeant markers, such as ruthenium red, and differentiate between pits that were topologically open or closed (Willingham et al.,

1981). This approach was developed into an elegant assay that defined different states of coated pit invagination according to the accessibility of the coated pit lumen to different molecular weight markers (Schmid and Smythe, 1991). However, biochemical approaches and studies of fixed material gave only indirect access to the crucial moments leading up to scission. More recently, pioneering work on exocytosis showed how live-cell fluorescence microscopy could be used to differentiate between intact exocytic vesicles (topologically closed) and those that had fused with the plasma membrane (topologically open) (Miesenbock et al., 1998). In this approach, GFP mutants, termed ecliptic phluorins (pHl) (Miesenbock et al., 1998; Sankaranarayanan and Ryan, 2000), that are nonfluorescent at pH 5.5 were targeted to the lumen of exocytic vesicles by fusion with VAMP2, a vesicular SNARE protein, where fluorescence was quenched by the acidic environment. The pH changes after exocytosis were thus signaled by a dramatic increase in vesicle fluorescence. In addition to monitoring the exocytosis of synaptic vesicles, it was found that the acidification of recycled vesicles was fast enough for the fluorescence signal to be a reliable index of synaptic vesicle endocytosis (Sankaranarayanan and Ryan, 2000). However, significant labeling of the plasma membrane with this protein (10%–15% on the plasma membrane) precluded the detection of single endocytic events. A later study tackled this problem by using photobleaching to eliminate this background and enabled measurements of single synaptic vesicle exocytosis and endocytosis, uncovering various modes of vesicle retrieval (Gandhi and Stevens, 2003).

Recently, a related approach was employed to resolve single scission events at CCPs in fibroblasts (Merrifield et al., 2005). Here, pHl was fused to the external domain of the constitutively endocytosed transferrin receptor (Tfnr) to generate Tfnr-phl. This construct was properly targeted to clathrin-coated structures at the plasma membrane and was competent for the binding and internalization of transferrin (Tfn). To visually isolate Tfnr-phl in coated vesicles from receptors on the plasma membrane, buffer at pH 5.5 was locally perfused onto the cell, and it rapidly, completely, and reversibly quenched all background fluorescence at the plasma membrane (Figure 3B). Since Tfnr-phl at the vast majority of clathrin-coated structures at the plasma membrane was quenched, these must have represented coated pits. However, Tfnr-phl at a minority of clathrin-coated structures was not quenched completely, and these acid-resistant spots must have represented isolated (i.e., topologically closed) vesicles. By alternating between locally perfused buffers of pH 5.5 and 7.4 in synchrony with image acquisition, the formation of individual coated vesicles could be monitored with a time resolution limited only by buffer exchange; in this case, this time was 2 s (Figures 3A and 3B). Scission was marked by an unambiguous binary signal, wherein a bright spot of Tfnr-phl fluorescence became visible in images acquired at pH 5.5 and colocalized with a cluster of Tfnr-phl (corresponding to a CCP) in images acquired at pH 7.4 (Figure 3C).

Most importantly, because scission could be detected without any preconceived notion of the dynamic signatures associated with CCV formation, this assay allowed the relationship between clathrin-coated structure dynamics and scission to be analyzed directly. It was shown that diffraction limited both clathrin-coated structures, probably corresponding to single coated pits, and larger clathrin-coated structures, corresponding to clusters of coated pits or flat clathrin lattices, could support multiple rounds of scission (Figure 3D). Thus, it was not possible, on the basis of clathrin-coated structure size, to use fluorescence microscopy to differentiate between endocytically active or inactive clathrin-coated structures. When a subpopulation of small, diffraction-limited coated pits was analyzed in detail, it was found that more than half of the scission events occurred without an appreciable change in coated pit fluorescence under EF illumination (d = 100 nm); these were termed “nonterminal events” (Figure 4A). Other scission events coincided with the complete disappearance of the attendant coated pit, and these were termed “terminal events,” since no further rounds of scission could occur once the entire coated pit had disappeared. The coincidence of terminal events (i.e., coated pit disappearance) and scission confirmed the previous assumption that coated pit disappearance is a valid marker for CCV formation (Gaidarov et al., 1999; Merrifield et al., 2002). Indeed, upon closer examination, it was found that terminal events were a good predictor of scission since 49% of the disappearing clathrin-coated pits coincided with scission events (our unpublished data). Since the scission assay can only detect half of all scission events (Figure 3A), this was very close to the theoretical prediction of 50% coincidence.

What were the biological correlates of nonterminal events? At present, three hypotheses can explain these observations (Figure 4B). In the first, local recycling of clathrin after scission may have occurred rapidly and locally, but it may have occurred below the detection of EF microscopy. Thus, although a coated vesicle formed and locally uncoated, a new coated pit reformed at the site of scission, and, to the observer, nothing appeared to change (Figure 4Ba). Second, it is possible that only a fraction of the coated pits and associated cargo invaginated and generated a coated vesicle (Merrifield et al., 2005), leaving the rest of the coated pits available for further rounds of coated vesicle creation (Figure 4Bb). Budding of coated vesicles from a larger clathrin-coated structure could be clearly seen in some instances (Blanpied et al., 2002; Gaidarov et al., 1999; Merrifield et al., 2005; Rappoport et al., 2003a; Yarar et al., 2005), but its incidence might be underestimated by the limited spatial resolution of light microscopy. Finally, the same coated pit could have undergone reversion between closed and open states (Figure 4Bc). This would be reminiscent of electrophysiological data showing that exocytic fusion pores could “flicker” between open and closed states (Breckenridge and Almers, 1987; Staal et al., 2004), and that the transient membrane neck produced during endocytosis was similarly unstable (Henkel et al., 2000).

As previously discussed, a combination of EF and Epi microscopy could be used to follow the inward movement made by CCSs during CME (Merrifield et al., 2002). However, although the inward movement of CCSs could be detected, it was not clear whether these

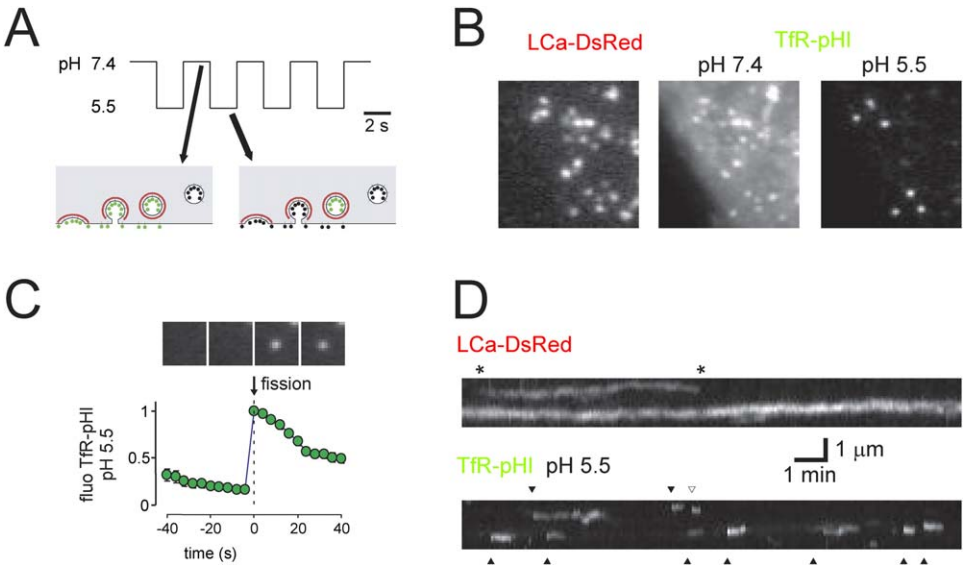

Figure 3. Method to Directly Visualize the Creation of CCVs in Live Cells

(A) Experimental protocol. A cell expressing LCa-DsRed (red lines) and TfR-pHl (green lollipops, fluorescent; black lollipops, quenched by protons) is alternatively bathed in solutions at pH 7.4 and pH 5.5. At pH 7.4, TfR-pHl on the plasma membrane and in nonacidic CCVs are visible. At pH 5.5, the fluorescence from nonacidic CCVs is isolated. A CCV formed at pH 7.4 will be visible on the next image at pH 5.5. CCVs formed at pH 5.5 will remain invisible. Thus, half of all CCVs created will be detected by this assay.

(B) Views of a Swiss 3T3 fibroblast expressing LCa-DsRed and TfR-pHl at pH 7.4 and 2 s later at pH 5.5. In a subset of CCSs, acid-resistant spots are clearly visible.

(C) The moment of scission can be precisely determined at the first frame, in which the CCV is visible. Four consecutive frames at pH 5.5, 4 s apart, of an example event are shown. The fluorescence centered around the CCP is measured for 103 events, normalized, and aligned to the moment of scission.

(D) Kymographs of two CCSs showing the formation of multiple CCVs by single CCSs. On top is a diffraction-limited CCP forming de novo (star), producing three CCVs (triangles), and disappearing (star). On the bottom is a larger CCS visible throughout the recording (15 min) that produced seven CCVs. See Merrifield et al. (2005) for a complete description of these data.

structures represented invaginating coated pits or coated vesicles moving into the cytoplasm after membrane scission (Santini and Keen, 2002). By simultaneously measuring coated pit movement and scission, it was shown that movement preceded scission, and that, on average, scission coincided with peak coated pit displacement from the plasma membrane (Merrifield et al., 2005). Therefore, the inward movement of coated pits measured in this and previous studies by using EF/Epi fluorescence microscopy must have corresponded to a late stage in coated pit invagination.

Earlier work also showed that the invagination of coated pits coincided with the recruitment of actin and proteins involved in actin polymerization (Benesch et al., 2005; Merrifield et al., 2002, 2004; Yarar et al., 2005). Thus, it was predicted that actin polymerization should begin before scission; and in an effort to test this hypothesis, the dynamics of cortactin, a dynamin and F-actin binding partner, were analyzed relative to membrane scission. It was found that cortactin-DsRed was recruited to only a minority of scission events (36%), although, when it did occur, recruitment began before scission over a similar time course to invagination. The low incidence of cortactin recruitment was somewhat surprising since, in previous studies, it was shown that ~80% of coated pit internalization events coincided with the formation of an actin plume (Benesch et al., 2005; Merrifield et al., 2002; Yarar et al., 2005). This may hint that the consortium of proteins recruited to scission events is not always the same, though whether this is related to the discovery of terminal and nonterminal scission events is currently unknown. A more general role for actin polymerization in fibroblast clathrin-mediated endocytosis was uncovered by using the G-actin-sequestering drug latrunculin, which blocked multiple aspects of clathrin-coated structure dynamics, including clathrin-coated structure formation, disappearance, merging, and splitting (Merrifield et al., 2005; Moskowitz et al., 2003; Yarar et al., 2005), as well as markedly reducing the incidence of scission by 80% (Merrifield et al., 2005). Remarkably, while a reduced number of scission events could still be detected in cells treated with latrunculin, these were invariably "nonterminal"-type events. Therefore, the disappearance of coated pits observed in terminal scission events may require actin polymerization.

Overall, to our knowledge, this study provides the first unbiased description of coated vesicle formation in live cells, and it has paved the way for a detailed molecular description of coated vesicle formation by using dual-color EF/Epi fluorescence microscopy.

Conclusion: Toward a Complete Molecular Description of CCV Formation

Although the use of live-cell imaging techniques for studying the molecular dynamics of clathrin-mediated

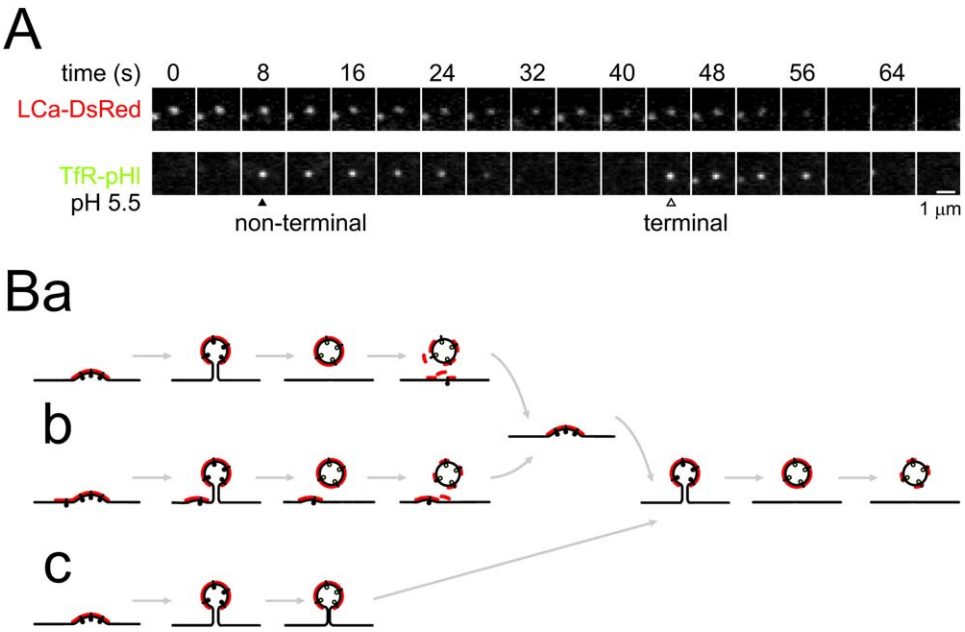

Figure 4. Three Alternative Scenarios for Nonterminal Events

(A) A CCP (labeled with LCa-DsRed) gives rise to two successive events, as detected with Tfr-phl at pH 5.5: a nonterminal event (black arrowhead), and, 36 s later, a terminal event (white arrowhead), where both markers disappear together.

(B) The late stages of invagination and membrane scission that could account for the observed nonterminal events, followed by a terminal event, are represented. Clathrin is red; Tfnr-phl is represented as lollipops, which are dark green when they are accessible to external protons and light green when they are protected from the ingress of protons. In (Ba), membrane scission proceeds as in the classical model of CME (Figure 1A), but recruitment of clathrin to the same plasma membrane spot occurs more quickly after membrane scission and vesicle uncoating. In (Bb), only a fraction of the CCP gives rise to a CCV, and a patch of clathrin is left on the plasma membrane, giving rise to another vesicle. In (Bc), after membrane scission, the vesicle is tethered to the membrane, and it eventually opens again. This newly opened vesicle may then close again, which would be seen as another scission event on the same CCP.

endocytosis is still in its infancy, this approach has both confirmed some features of the classical model of clathrin-mediated endocytosis and provided some new and exciting insights. One of the most striking features of data published thus far is that proteins fall into natural groups based on their dynamic relationship to clathrin-coated structures in both mammalian cells and yeast. In the future, unbiased assignment of proteins to different groups, based on their recruitment profiles relative to coated pit invagination and scission, may yield valuable clues concerning the function(s) of specific proteins and a more detailed insight into the regulation and control of clathrin-mediated endocytosis. This work may be elaborated upon by measuring changes in the kinetics of the recruitment/dismissal profiles of different proteins in response to intervention, such as the introduction of mutations (Benmerah et al., 1999) or the knockdown of binding partners with siRNA (Engqvist-Goldstein et al., 2004). As these tools are perfected, they may be used to target some old problems. For instance, it is not entirely understood how the site of coated pit formation is chosen, if it is chosen at all (Ehrlich et al., 2004). Nor are the signals that trigger the final stages of invagination and scission during clathrin-mediated endocytosis fully understood, although it seems likely that PIPs and phosphorylation play a role. Also, how actin polymerization is triggered and integrated into the mechanism of invagination and scission during clathrin-mediated endocytosis is not entirely clear.

To achieve these goals, a considerable effort will have to be made to increase the throughput of live-cell imaging studies to keep pace with the detailed biochemical characterization of endocytic proteins and lipids. This will include improvements in the imaging technology used, improvements in the analysis algorithms deployed to process and handle the large amounts of data produced, and the continued development of novel optical assays to measure and quantify molecular dynamics during CME. If these challenges are met, then it seems likely that the study of clathrin-mediated endocytosis will remain at the vanguard of technological innovation in cell biology for some years to come.

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