Cell. Mol. Life Sci. (2009) 66:2049–2065
DOI 10.1007/s00018-009-0001-y

Cellular and Molecular Life Sciences

REVIEW

Functions of actin in endocytosis

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Received: 19 January 2009 / Revised: 10 February 2009 / Accepted: 13 February 2009 / Published online: 17 March 2009

Abstract Endocytosis is a fundamental eukaryotic process required for remodelling plasma-membrane lipids and protein to ensure appropriate membrane composition. Increasing evidence from a number of cell types reveals that actin plays an active, and often essential, role at key endocytic stages. Much of our current mechanistic understanding of the endocytic process has come from studies in budding yeast and has been facilitated by yeast's genetic amenability and by technological advances in live cell imaging. While endocytosis in metazoans is likely to be subject to a greater array of regulatory signals, recent reports indicate that spatiotemporal aspects of vesicle formation requiring actin are likely to be conserved across eukaryotic evolution. In this review we focus on the 'modular' model of endocytosis in yeast before highlighting comparisons with other cell types. Our discussion is limited to endocytosis involving clathrin as other types of endocytosis have not been demonstrated in yeast.

Keywords Actin · Dynamin · Endocytosis · Myosin · *Saccharomyces cerevisiae*

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Introduction

Endocytosis is the process whereby plasma membrane is internalized into the cell and pinched off to form a vesicle which can then fuse with endosomes and enter the endo-lysosomal system. Endocytosis is a broad term that can pertain to all mechanisms by which a cell internalizes material from outside its plasma membrane. Within the context of this review we are focusing on the process of clathrin-mediated endocytosis, which is the best studied form of endocytosis and a process highly conserved from yeast to humans. The process of clathrin-mediated endocytosis has been studied for decades and is characterized by the formation of a protein coat of clathrin and other adaptors around the forming vesicle. The clathrin/adaptor protein coat is disassembled from the vesicle before the vesicle moves into the cell to eventually fuse with an endosome. Cargo can be trafficked from early endosomes to late endosomes and lysosomes for degradation, or recycled back to the plasma membrane ([1]; reviewed in [2–4]).

Increasing evidence from studies involving a number of cell types has highlighted the importance of the actin cytoskeleton during endocytosis. It now appears that rather than being a passive barrier that must be disassembled for vesicle internalization, actin can be considered to play an active role in assisting and even driving certain stages of the endocytic process. The majority of our insight into the role of actin in endocytosis has come from studies on the budding yeast *Saccharomyces cerevisiae*. Although endocytosis in mammalian cells is more complex and subject to a wider array of regulatory mechanisms, recent technological advances have enabled the presence of actin and actin-binding proteins to be demonstrated at endocytic sites. In addition, real-time imaging indicates that

spatiotemporal aspects of actin recruitment and the formation of endocytic vesicles are likely to have been conserved across eukaryotes.

In this review we discuss the recent advances that have been made in our understanding of the role of actin in endocytosis and highlight how actin is regulated to allow the formation and movement of endocytic vesicles from the plasma membrane. The largest part of this review focuses on the abundance of data recently obtained from studies on yeast that forms the basis for most of our current understanding of the role of actin during endocytosis. We then consider the most recent advances that demonstrate possible roles of the actin cytoskeleton in endocytosis in mammalian cells.

Actin and endocytosis in yeast

Defining the link between actin and endocytosis

Actin organization in yeast was first described over 20 years ago when Adams and Pringle identified two major actin structures in yeast: cortical actin patches and actin cables [5]. Since this time the primary role of both actin cables and patches has been largely elucidated. Cables, which run along the mother–bud axis, are considered to be 'tracks' along which organelles and secretory vesicles can be trafficked to the growing daughter cell, while actin patches are now considered to be sites of endocytosis. The latter are the focus of a large part of this review (Fig. 1a; Table 1). While actin patches were observed to localize to regions of active cell growth, their role at these sites remained unclear for many years. The first evidence for a link between actin and endocytosis was from a number of genetic screens for yeast endocytic (*end*-) mutants [6–9]. Upon detailed analysis, some of these *end* mutants were found to correspond to previously identified actin-associated proteins (*end4* = *sla2*; *end5* = *vrp1*; *end6* = *rvs161*; *end7* = *Act1*), while others were unknown but caused defects in actin organization (*end3*; [10]). It was also shown that endocytic internalization of the pheromone α-factor was blocked in strains with mutant actin alleles or by the absence of an actin bundling protein, Sac6, also known as fimbrin [11]. Other actin-associated proteins were also found to be involved endocytosis when it was shown that deletion of the *S. cerevisiae* type I myosins (actin-dependent motor proteins) *MYO3* and *MYO5* caused defects in endocytosis [12]. Further evidence for the actin–endocytosis link came from studies with cell-permeant drugs affecting actin. Latrunculin-A, a drug which sequesters actin monomers, was shown to block vesicle internalization [13–15]. Subsequent experiments used jasplakinolide, a drug which stabilizes F-actin and causes it to accumulate as a large F-actin clump in the cell. One such study demonstrated that the presence of filamentous (F-) actin alone was insufficient for endocytosis to occur and that the actin also had to be dynamic [16].

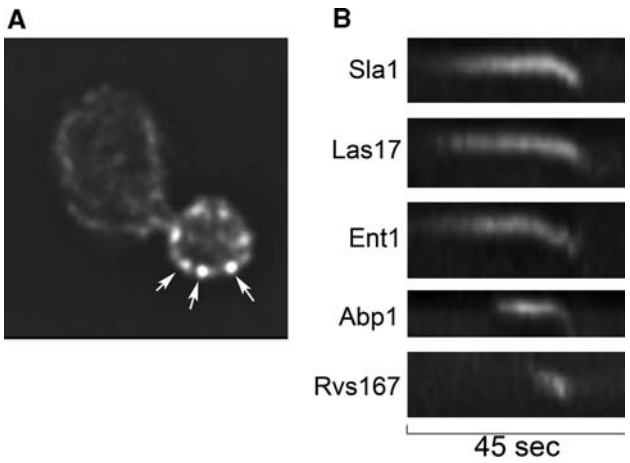

Fig. 1 Actin in *Saccharomyces cerevisiae* and the analysis of endocytic actin patches. **a** Actin is visualized in budding yeast cells using rhodamine phallodin. Two main structures are visualized, cortical actin patches (*spots* marked with *arrows*) and actin cables that run along the mother bud axis. **b** Kymographs generated from movies to show the behaviour of different proteins that assembly and disassemble from the endocytic complex. Sla1, Las17 and Ent1 arrive relatively early in the process and remain non-motile for the majority of their lifetime. Abp1 is used as a marker of actin's arrival at the complex, shortly before invagination is observed. Rvs167 is an amphiphysin thought to be involved in scission; it arrives shortly after inward movement has commenced

Over the last 20 years more than 60 proteins have been proposed to function in endocytic internalization, or to have a localization or interactions consistent with involvement in this process in yeast. Significant insights into the kinetic behaviour of actin came from studying actin patch proteins in real-time [17–19], which revealed that a patch is formed at the plasma membrane and that, after a few seconds, it moves rapidly into the cell. However, such studies also revealed that other proteins regulating actin appeared to have distinct behaviours, often remaining relatively non-motile at the membrane or showing just small inward movement [19] (Fig. 1b). Despite this knowledge and the identification of many of the proteins present at the cortical sites, progress in our understanding of the endocytic mechanism and the involvement of actin was limited until two key technological advances were combined. One was the advent of spectral variants of green fluorescent protein (GFP), which meant that the relative timing of the localization of two individual proteins could be analysed. The second was improvements in live cell imaging capabilities. These approaches were first combined in an elegant set of experiments conducted by Kaksonen et al. [20] and led to

Table 1 Endocytic and actin regulatory proteins in yeast endocytosis and known mammalian homologues

Yeast protein	Proposed function	Mammalian orthologue	References
Endocytic coat module			
Clathrin heavy chain (Chc1)	Stabilization of membrane curvature	Clathrin heavy chain	[15, 20, 34]
Clathrin light chain (Clc1)	Binding clathrin and recruiting other coat proteins	Clathrin light chain	[36]
Ent 1	Binds clathrin, part of coat complex	Epsin	[28, 33]
Ent 2	Binds clathrin, part of coat complex	Epsin	[15, 33]
Ede1	Binds ubiquitin and Ent proteins	Eps15	[26]
YAP180/1	Clathrin coat assembly, binds clathrin and Pan1	AP180/CALM	[33]
YAP180/2	Clathrin coat assembly, binds clathrin and Pan1	AP180/CALM	[129]
Sla1	Cargo binding, Las17 inhibition	CD2AP/CIN85	[19, 29, 38]
Pan1	Binds Clc1, Yap180 and may activate Arp2/3	Intersectin	[14, 130]
End3	Binds Pan1 and recruits Sla1	–	[10, 19]
Sla2	Coupling actin to vesicle coat	HIP1R	[35, 36]
Actin nucleation and polymerization			
Arp2/3	F-Actin nucleation and branching	Arp2/3	
Las17	Activator of Arp2/3, generates loose F-actin network prior to myosin recruitment	WASP	[21, 49]
Myo3, Myo5	Arp2/3 activation and actin motor drives invagination and possible role in vesicle scission	Type I Myosin	[12, 42, 43]
Bbc1	Inhibitor of Las17		
Sla1	Adaptor protein, cargo binding, regulator of Las17	CD2AP/CIN85	[19, 29, 38]
Vrp1 (verprolin)	Regulation of type I myosins.	WIP	[42, 44]
Bzz1	Las17 and myosin 1 interactor	Syndapin	[24]
Ysc84	Interacts with Las17 and increase rate of actin polymerization	SH3yl-1	[50]
F-actin regulation			
Cap1, Cap2	Funnelling monomer to growing actin filament ends	Capping protein	[14, 53]
Sac6	Bundling and cross-linking actin	Fimbrin	[11, 22, 53]
Scp1	Bundling and cross-linking actin	SM22/transgelin	[22, 56]
Cofilin (cof1)	Depolymerizing actin filaments	Cofilin	[52, 57]
Other Proteins			
Abp1	Binding actin to coat, and recruiting regulatory kinases Ark1, Prk1	mAbp1	[14]
Rvs161/Rvs167	Vesicle scission	Amphiphysin	[14, 65]
Vps1	Sla1 interaction, actin organization	Dynamin	[61]
Arf3	Regulator of PtdIns(4,5)P2 levels and regulator of actin	Arf6	[81, 82]
Ark1, Prk1	Kinases regulating disassembly of endocytic coat	AAK, GAK	[131, 132]

the first real understanding of the links between actin and the endocytic machinery. Using real-time fluorescence microscopy with GFP- and red fluorescent protein (RFP)-tagged pairs of proteins, these researchers were able to place actin at the centre of the endocytic process. In essence, they revealed that endocytosis involves the sequential assembly and disassembly of proteins at a site on the plasma membrane. Actin recruitment occurs at a

defined stage of this process that corresponds to the invagination and subsequent movement of the vesicle into the cell. By investigating activators of the Arp2/3 complex, the main actin nucleator, Kaksonen et al. were also able to define a pathway where a sequence of changes in cortical patch composition correlates with changes in patch motility [20].

In a second study, Kaksonen et al. [14] went on to analyse the effect of 60 gene deletions of non-essential putative endocytic or actin-regulatory proteins, and through this work they revealed new functions for several proteins at the endocytic site. The laboratories of David Drubin and others have subsequently added to this core data to formulate a clear mechanism through which it has become apparent that the organization and regulation of actin is integral to the endocytic process [14, 15, 20–25].

As depicted in Fig. 2a, the process essentially involves distinct stages of non-motile, slow-moving and fast-moving patches that can be defined by the presence of discrete protein modules. The four main functional modules that assemble at endocytic sites in a co-ordinated and sequential manner are: the endocytic coat complex, the actin polymerization machinery, the actin dynamic regulation module and the vesicle scission proteins. Our current understanding of these modules and the role of actin at the corresponding stages of endocytosis will be discussed in turn.

Stage 1: assembly of the endocytic coat complex

This is the earliest stage of endocytosis and involves a number of proteins that become associated at the plasma membrane. Recruitment of these proteins does not appear to involve actin, as disruption of actin with latrunculin A does not impair their localization to the plasma membrane. The coat complex has a number of key functions: first, it is involved in cargo recruitment; second, it is likely to cause a degree of membrane curvature that will facilitate the invagination process; third, it harbors the proteins that will trigger actin polymerization. Real-time images of these components of endocytosis are visualized as a non-motile patch that later moves a small distance into the cell. It has been proposed that this slow movement occurs during the invagination and scission steps, after which these proteins disassemble from the vesicle.

It is currently unclear which factors are required on the plasma membrane to initiate complex formation. In general, however, the first proteins to localize at the site include clathrin, Ede1, the epsin homologues Ent1 and Ent2 and the AP180/CALM homologues (YAP1801/2). Ede1 is an adaptor-type protein that is reported to localize to membranes in a ubiquitin-dependent manner, and ubiquitin, in turn, is known to be an important signal for

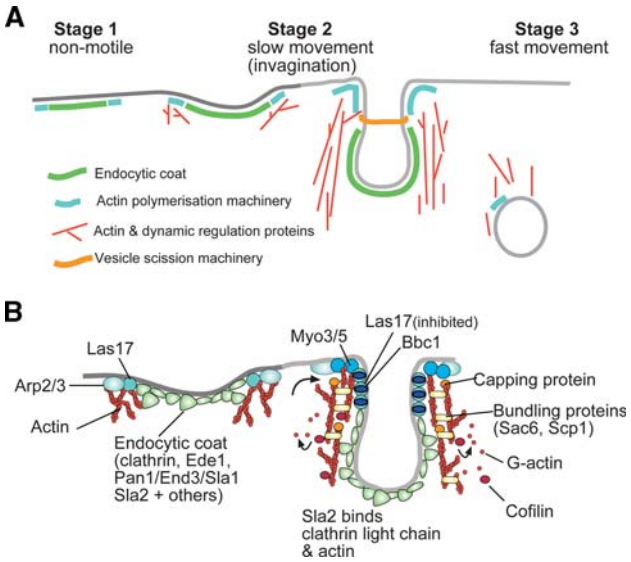

Fig. 2 Schematic depicting stages defined in yeast endocytosis. **a** Work by many laboratories has led to the development of a general model for the key stages of the endocytic process in *S. cerevisiae*. Stage 1 defines the phase during which the endocytic coat becomes assembled at the plasma membrane. Embedded within this structure, but held inactive, are proteins important for the initial stages of actin polymerization. Stage 2 is the slow movement or invagination stage. This corresponds to the time during which actin is polymerizing and forming a framework necessary to ensure the inward growth of the membrane. Stage 3 represents the scission and movement of the newly formed vesicle away from the membrane. The rate of movement is increased following scission. **b** Actin regulatory proteins are essential for invagination, as is the function of actin. During this stage actin organization is regulated by many proteins. Initially, the WASP homologue Las17 plays a key role in driving Arp2/3-dependent polymerization to form a loose network of F-actin at the site. This F-actin is required to recruit strong actin polymerization factors—the type I myosins. During the invagination stage, Las17 is possibly held inactive by a protein Bbc1. Dynamic regulation of actin filaments requires the activity of capping protein to ensure the funnelling of new actin monomer to filament ends and cofilin to drive depolymerization of actin. Actin bundling proteins Sac6 and Scp1 function together to form higher order F-actin structures, such as bundles or a cross-linked mesh, that provide a strong framework necessary to support inward growth of membrane

mediating endocytosis [26, 27]. Furthermore, deletion of *ede1* combined with inactivation of the ubiquitin-interacting motif of Ent1 causes a significant endocytic defect [28]. These data suggest that ubiquitination may serve as a signal to trigger the formation of endocytic complexes at the appropriate sites. However, ubiquitin is not the only signal known to be associated with cargo uptake, and other proteins may also be involved in site selection, such as Sla1, which binds to the motif NPFXD found on various cargoes, including the pheromone receptors Ste2 and Ste3 and the P4-ATPases Drs2 and Dnf1, which regulate the lipid composition of vesicles [29, 30]. Localization of clathrin and adaptor proteins is followed by the recruitment of Sla2 (homologue of HIP1-R) and then by recruitment of a

module containing three proteins, Pan1, End3 and Sla1 [15, 20]. Several of these proteins (including Ent1 and Sla2) contain ENTH or ANTH domains, which are known to interact with phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2]-containing membranes to induce curvature [31–33].

The role of clathrin in yeast endocytosis has been somewhat controversial as the deletion of clathrin does not completely abrogate endocytosis. However, analysis of endocytic patches in clathrin deletion strains reveals that clathrin contributes significantly to the total number of endocytic sites that are observed and also to the stability of the endocytic complex during invagination [15, 20]. Whether clathrin forms a basket-like meshwork around the vesicle also remains unresolved. However, given the profile of endocytic invaginations in yeast as tubules rather than more rounded vesicles, and the localization of clathrin to a patch at the distal tips of these, it seems most likely that clathrin is largely involved in stabilizing the curvature at the tip of the invagination during inward growth [34].

There are at least three key proteins in the endocytic coat complex that are associated with actin. Sla2 binds the clathrin light chain and also binds actin through its talin homology domain [35, 36]. The interaction between Sla2 and the actin machinery appears to require the presence of the Sla1 protein [37]. These and other data indicate that Sla2 is potentially a central player in linking the growing actin meshwork to the endocytic coat during the invagination stage (see below). As well as binding to Yap1801/2 and End3, Pan1 is reported to be an activator of Arp2/3 and thereby drives actin polymerization, although the exact stage at which this function of Pan1 is involved in endocytosis is not yet fully understood. It has been proposed that Sla1, in addition to binding to endocytic coat components and cargo proteins, regulates actin. Deletion of *SLA1* has two key effects—lack of uptake of the NPFXD cargoes and an alteration in actin dynamics; for example, the cortical actin patches become much larger, indicating that actin polymerization may be over-activated [38, 39]. The most likely explanation for the changes in actin is that Sla1 binds to and inhibits a key regulator of Arp2/3 that associates with the endocytic coat [40]; this protein is Las17, the homologue of mammalian WASP. Release of Sla1 inhibition of Las17 is potentially a key step in the activation of actin polymerization and leads to the invagination step of endocytosis. Thus, while the coat complex forms independently of actin, its movement and disassembly require growth of an actin filament network.

One additional important insight has emerged from recent studies carried out by researchers at the Cooper laboratory [21, 41]. They used spinning disc confocal microscopy to analyse forming endocytic patches and observed that during endocytic site formation, the endocytic coat complex undergoes a series of apparently random movements confined to a small area and that it appears to be tethered or corralled while an actin meshwork is assembled. Only after this stage is movement away from the membrane permitted. These observations suggest that actin filaments are generated and play a function prior to the invagination step. The function of the filaments is not known, but one could postulate that they function in the localization of the type I myosins, as it is known that Myo3 and Myo5 (but not Las17) require F-actin for localization. Alternatively, (or additionally), these filaments may play a meshwork role in stabilizing the pit and committing it to endocytosis.

Stage 2: invagination

The slow movement stage of the cortical endocytic complex is proposed to correspond to the invagination stage of endocytosis, involving growth and extension of the membrane into the cell. Associated with this step is the initiation of actin polymerization by Arp2/3 and a number of actin nucleation promoting factors (NPFs), the dynamic regulation of actin filaments and the increased curvature of the membrane. Both lipids and lipid-interacting proteins are likely to be critical to this process. One suggestion is that this slow movement is a reflection of the rate of actin polymerization, with actin monomers being added at the plasma membrane rather than on the vesicle [14].

Actin network growth

The actin network growth machinery comprises the NPFs Las17 (yeast WASp) and the type I myosins Myo3 and Myo5 as well as a protein required by the myosins for their NPF activity, verprolin (Vrp1), which is the yeast WIP homologue [23, 42–44]. Two other proteins, Pan1 and Abp1, have been reported to display activation of Arp2/3 in vitro, but the level of activity of these proteins is much lower than either Las17 or type I myosins [45, 46]. More recent data indicate that deletion of the NPF activity of Abp1 or Pan1 does not have a marked impact on actin polymerization or endocytic invagination in vivo [25]. Two important regulators of the Arp2/3-activating function of the NPFs are Sla1, which is part of the endocytic coat and an inhibitor of Las17, and Bbc1, which may inhibit the activity of Las17 and Myo5. Interestingly, Las17 and the myosins have very different kinetic properties at the cortical patch: Las17 arrives early and remains immotile on the cell surface for 10–20 s before any invagination is observed; verprolin arrives several seconds after Las17, while Myo3 and Myo5 arrive immediately prior to invagination. All of these proteins remain at the plasma membrane during invagination (i.e. they do not show

inward movement with the endocytic coat proteins). This localization of the NPFs indicates that inward movement is driven by actin polymerization at the plasma membrane rather than on the surface of the forming vesicle [20]. The nucleation of actin filaments on the plasma membrane rather than on invaginated vesicles was also demonstrated using fluorescence recovery after photobleaching (FRAP). Following photobleaching of a region of the plasma membrane containing actin patches, recovery was observed first at the plasma membrane-side of the complex rather than further down the invagination [20]. The Las17 inhibitor Sla1 is closely associated with Las17 during the non-motile phase of endocytosis. However, once invagination initiates, Sla1 moves in at the tip of the invagination with the other coat proteins. The inhibitor Bbc1 arrives with the type I myosins and appears to associate with the non-motile complex during the majority of the invagination stage [14, 34]. At the end of the slow movement stage, the type I myosins and Bbc1 disassemble from the site. A recent report has shown that in longer invaginations, Las17 can be detected at the site of scission, part way down the invagination [34]. This result suggests the possibility that Las17 could play a role later in coupling actin dynamics to vesicle movement away from the plasma membrane following scission (see below).

Deletion studies of different NPFs, or NPF inhibitors, clearly demonstrate their importance in endocytosis. Strains lacking only Las17 show almost no inward movement of endocytic reporter proteins. The loss of one of the type I myosins (Myo5) decreases the probability that a patch will undergo inward movement, while the loss of both type I myosins (Myo5 and Myo3) also results in patches showing little or no movement away from the cortex [21, 23, 25]. Vrp1 is localized by Las17 but appears to be critical for the function of the type I myosins. Deletion of Vrp1 also abrogates the majority of invagination that can be observed [14, 44]. These various results define a clear role for NPFs in driving actin polymerization and thereby membrane invagination.

Recent studies have attempted to address two key questions relating to the role of the NPFs at the endocytic site. First, do Las17 and the myosins play distinct roles at the site and, if so, at which stage of endocytosis does each function? Second, what are the relative contributions of the NPF activity and the motor domain to type I myosin function in endocytosis? Two studies have investigated the effect of deletion of the domains of Las17 or Myo3/5 that interact with Arp2/3 to activate its nucleation properties. Sun and colleagues [25] deleted the WH2 (WASp homology domain 2—actin monomer binding) and the acidic domain that binds Arp2/3, while Galletta et al. [21] deleted only the acidic domains of relevant proteins. Overall, however, the conclusions of both groups were very similar in that a *las17Δacidic* mutant displays a delay in polymerizing actin at a very early stage of endocytosis while the invagination stage was relatively normal. In contrast and somewhat surprisingly, deletion of the *myo5Δacidic* or *myo3Δacidic* regions alone or even in combination had little or no phenotype [21]. Combinations of mutants had markedly stronger phenotypes than single mutants, with a triple *Las17Δacidic*, *Myo3Δacidic*, *myo5Δacidic* mutant showing defects both in patch movement and time spent at the origin prior to inward movement. The deletion of Vrp1 did not block actin polymerization, but it did prevent inward movement. Together, the results suggest that the Arp2/3 binding regions of Las17 and type-1 myosins have a significant level of redundancy and that in the absence of the domains to activate Arp2/3, an actin network with sufficient branch density or filament length cannot be constructed. In addition, the data indicate that Las17 is likely to function prior to any invagination, while the type I myosins may play a more important role during invagination.

An interesting observation from these carefully analysed studies is that deletion of the Arp2/3 interacting domain in these proteins had a markedly less severe phenotype than might have been predicted, suggesting that other interactions of Las17 and Myo3/5 are contributing important functions to the overall endocytic process. Las17 has a plethora of known binding partners, and it is likely that these interactions may contribute to the Las17 activation of Arp2/3 as well as to other functions of this key endocytic protein [47–49]. Most recently, a cortical patch protein, Ysc84, has been shown to bind to actin and to increase its polymerization rate in the presence of Las17. This interaction would provide a potentially Arp2/3-independent route for driving actin polymerization during endocytosis [50]. Myo5 has been studied further in terms of the relative contribution of its motor domain and NPF activity to endocytosis [25]. These elegant biochemical and correlative in vivo studies demonstrated that the motor domain and NPF activity are functionally separable. Analysis of motor domain mutants has revealed that the motor domain is not required for actin polymerization at the endocytic patch per se but that it is essential for the inward movement corresponding to invagination. Furthermore, there is no movement of the actin filaments inward in a Myo5 motor domain mutant, suggesting that the type I myosin motor domains function to translocate actin filaments away from the membrane, thus providing the force to drive the endocytic bud into the cell.

A final, but very important consideration is the trigger for actin polymerization at an endocytic site. One scenario is that the inhibition of Las17 by Sla1 must be first lifted and that this lifting will then trigger the onset of actin polymerization and consequent invagination. However, to

date, little is known about this critical step in the process. Some in vitro studies [25] have shown that Sla1 inhibition can be removed in a dose-dependent way by a syndapin homologue, Bzz1. However, deletion of Bzz1 would then be expected to prolong the timing of Sla1 inhibition and delay endocytosis, and this is not observed in vivo. Another possibility is that Sla1 binding to its cargo NPFXD domain could cause a conformational change to release inhibition. This presents a situation in which endocytosis may be linked to the binding of appropriate cargoes, but as yet this has not been demonstrated.

Actin dynamic regulation

Following the initiation of actin polymerization, many other actin-binding proteins become associated with the cortical endocytic complex (Fig. 2b). Several of these are involved in regulating the organization and turnover of actin filaments while others, such as Abp1, localize to actin possibly in order to recruit further regulatory proteins. The proteins in this module include yeast Capping Protein Cap1/2p, the bundling proteins Scp1 and Sac6 and the depolymerizing complex Aip1/cofilin. All of these proteins are dependent on actin for localization, but unlike the coat proteins, they show some co-localization with the vesicle as it begins to move into the cell [20, 22, 51, 52]. Cap1 and Cap2 function as a heterodimer to cap the barbed end of growing actin filaments and represent the α- and β-subunits of the yeast capping protein, respectively [53]. Early studies on capping protein mutants indicated no defects in endocytosis. However, the advent of live cell imaging, which permits a more sensitive analysis of the invagination process, has now placed this protein firmly in the centre of actin dynamic regulation during the invagination stages of endocytosis. Deletion of capping protein genes was observed to significantly reduce the rate of inward patch movement, leading to a particularly prolonged stage 2 lifetime. Given the known biochemical activity of capping protein, it has been proposed that it may function to funnel monomeric actin to the barbed ends of growing filaments. In its absence, there is less monomeric actin being delivered, and so the process of invagination is slowed [14, 41]. More recent analysis has determined requirements for capping protein in both invagination and post-scission movement, although its role at the former stage appears to be more important.

Sac6 (yeast fimbrin) has a long history associated with both actin organization and with endocytosis in yeast [11, 54]. In vitro, Sac6 bundles actin filaments, and in vivo, its deletion destabilizes the actin cytoskeleton [13, 54]. Unlike the majority of yeast actin binding proteins, it associates with both actin cables and cortical actin patches. However, it was not until the effects of its deletion on endocytic reporters Sla1GFP and Abp1RFP were reported that its role in endocytosis became more apparent. In the absence of Sac6, endocytic patches show a very poor rate of internalization, with many patches showing little or no movement away from the membrane [14]. Thus, not only is actin polymerization important for invagination, but also the organization of these filaments into higher order meshworks and bundles is crucial if a patch is to be internalized. Sac6 is not, however, the only actin-bundling protein in cortical patches, and other studies have investigated the function of a second bundling protein, Scp1, the yeast homologue of SM22 or transgelin. Scp1 localizes to actin patches and, in vitro, organizes actin into tight bundles [55, 56]. Although its deletion does not appear to disrupt actin organization or the uptake of fluid phase markers of endocytosis, it does significantly exacerbate both actin and endocytic phenotypes of a *sac6* null strain, indicating significant overlap of function [55, 56]. More recently, detailed analysis of the effects of deletions of these bundling activities on endocytic reporters demonstrated that both single deletions prolong the lifetime of the endocytic patch to a similar degree, although patches were more likely to invaginate successfully in the *Δscp1* strain. In combination, *Δscp1Δsac6* cells showed almost no endocytic invagination and very prolonged patch lifetimes. Interestingly, Sac6 appeared to function more in the invaginating stage and Scp1 at later stages, potentially post-scission [22].

Cofilin has been widely studied in many cell types and, in all cases when tested, it has been shown to be essential. In *S. cerevisiae*, cofilin has been shown to associate with cortical actin patches, and its role in the disassembly of actin filaments is required for normal endocytosis [57, 58]. Since these studies, a second protein Aip1 has been shown to associate with cofilin within the patches and, in vitro, Aip1 modulates the actin-depolymerizing function of cofilin [59]. However, it was only very recently that live cell studies have been undertaken to investigate the behaviour of cofilin in endocytosis in real time. The problem had been that neither–nor C-terminally tagged cofilin were functional. This has now been circumvented by the incorporation of GFP into the sequence of cofilin (between N74 and G75) with flanking 12 amino acid linkers [52]. Okreglak et al. have now shown that GFP cofilin is recruited to patches several seconds after initial actin assembly and associates with the patch through the invagination and disassembly stages. Cofilin localization is not found immediately adjacent to the plasma membrane, indicating that it does not associate with newly formed filaments. Despite previous reports of Srv2/CAP and Aip1 contributions to the function of cofilin [59], Ogrelak and colleagues also report that, in their study, neither Srv2 nor Aip1 appeared to contribute to the cofilin-dependent actin

subunit flux in the mutant model examined. This study also revealed that a cofilin mutant (cof1-22) was able to internalize material, albeit with slightly delayed kinetics, but was significantly impaired in the timely disassembly of actin from the internalized membranes. Overall, these new data demonstrate that cofilin is the main player maintaining the required rate of actin monomer flux throughout the endocytic actin network.

Stage 3: vesicle scission and movement away from the plasma membrane

While the mechanistic role of actin in driving invagination is clearly critical, actin also plays roles in the subsequent stages of endocytosis. Indeed, there appears to be a requirement for actin in vesicle scission, coat disassembly and in the trafficking of the newly formed vesicle away from the plasma membrane. During scission and coat disassembly, the role of actin might be regarded as passive insofar as its presence ensures that key regulatory factors are appropriately recruited. In vesicle trafficking, it seems most likely that actin plays an active role in driving movement.

Vesicle scission

In mammalian cells, the role of vesicle scission has been ascribed to the large molecular weight GTPase, dynamin. Dynamin is a multi-domain protein with a C-terminal proline-rich domain that enables it to interact with a variety of Src homology 3 (SH3) domain-containing proteins, several of which have been implicated in endocytosis [60]. In yeast, the most likely dynamin homologue to play a role in scission is Vps1. Vps1 has been shown to interact with the endocytic adaptor protein Sla1, and its deletion does cause defects in actin organization [61] although no defect in endocytic internalization has been reported [62]. The role of vesicle scission in yeast endocytosis is considered to involve the amphiphysin module**.** There are two amphiphysins that form a heterodimer, comprising Rvs161 and Rvs167. These proteins localize transiently to endocytic sites following actin polymerization. In patches they show an inward movement of about 100 nm, which is thought to represent the point of vesicle fission, after which they disassemble [14]. Scission is made possible by Rvs161p and Rvs167p, both containing BAR domains that have been shown to bind and tubulate membranes in vitro [63]. Rvs167 has been shown to interact with, or be in complexes with a large number of other endocytic proteins, including actin itself, Las17, Myo5, Sla1 and Sla2 [43, 49, 64, 65]. It has been proposed that some of these interactions are mediated through its SH3 domain. Whether the association with actin is direct or via actin binding proteins is not yet known, however, it is clear that the amphiphysin has very close association with actin during endocytosis. Scission has also been observed to still occur in the absence of the amphiphysins, albeit at a lower rate, suggesting that other proteins are somehow involved. A possible role for type I myosins in scission was shown when a point mutation in the myosin-I lipid binding region caused the accumulation of long invaginated endocytic tubules in yeast—possibly due to failed scission [23]. More recently, electron microscopy dual-labelling experiments with actin, Rvs167 and the type-I myosin Myo5 in mature endocytic profiles revealed distinct sites of actin and myosin I localization either side of the amphiphysins [34]. This observation was consistent with unpublished observations that type-I myosins have a co-operative role with the scission machinery [34]. One proposal is that the actomyosin structure forms a contractile structure round the invaginating vesicle neck to cause scission—similar to the current model for dynamin-dependent scission in mammalian cells [60]. Another possibility is that the actomyosin structure places tension on the neck from either side, allowing the amphiphysins to tubulate the membrane and cause vesicle scission [66]. Observations that actin forms a spiral arrangement around plasma membrane invaginations [67] and that type-I myosins can slide along actin filaments could support either of these hypotheses [34].

Actin and coat disassembly

Analysis of forming endocytic vesicles suggests that scission and coat disassembly occur at about the same time, but the exact timing of events is not yet clear. The most likely scenario is that coat disassembly is concomitant with, or occurs immediately after the scission event. Removal of the coat is required for fusion at the endosome. Two key proteins involved in disassembly are the Ark1/Prk1 kinases, which are related to AAK and GAK in mammalian cells [68]. Ark1 and Prk1 are recruited to the endocytic complex through interactions with the SH3 domain of Abp1. Using GFP tags, it has been shown that Ark1p and Prk1p arrive at patches after the commencement of actin assembly and coat internalization. Deletion of the *S. cerevisiae* actin-regulating kinases Ark1p and Prk1p causes a failure in the disassembly of endocytic complexes, and the latter persist in the cytoplasm [69]. Deletion of Abp1 also causes coated vesicles to persist longer in the cytoplasm although this phenotype is much less severe than that caused by the deletion of both kinases, indicating that these proteins can potentially phosphorylate their substrates without prelocalization to the complex. There are several known substrates for these kinases within the endocytic coat complex, including Pan1, Sla1 and the epsins Ent1 and Ent2. Phosphorylation of these proteins

has been demonstrated to be coupled to their disassembly from the invaginated vesicle [70, 71].

A second family of proteins involved in coat disassembly are the synaptojanins, which are phosphaditylinositol-4,5-bisphoshpate phosphatases, with Inp52/Sjl2 appearing to be most important in this role [72]. These enzymes mediate dephosphorylation of PtdIns(4,5)P2 to PtsIns 4-P, which is likely to reduce the affinity of certain coat components for the vesicle membrane. In particular the ENTH and ANTH domain-containing proteins Sla2 and Ent1/2 will likely lose their affinity for the membrane and disassemble from the site [31, 32, 73]. Toret and colleagues have recently suggested that different complexes within the coat are disassembled through these distinct pathways [74], with Ark1/Prk1 being responsible for the disassembly of the Sla1/Pan1/End3 complex while Inp52/Sjl2 is responsible for the removal of Sla2 and the epsins. As recruitment of these disassembly complexes is actin-dependent, it may be viewed as a negative feedback loop in which actin assembly leads to invagination of the endocytic coat and membrane and also to recruitment of the factors that will lead to coat disassembly and scission.

Actin in vesicle movement

Following scission, the vesicle is uncoated and moves away from the membrane to fuse with early endosomes. Vesicles travel at varying speeds that can reach about 250 nm/s. In some cases, trajectories appear undefined, while in other cases, there appears to be a fairly linear movement. While actin appears to be important for movement to endosomes, there are two main roles that it may play. First, actin cables (bundles of actin filaments) could serve as tracks to guide the vesicles into cells. Second, the vesicle could undergo a comet-tail type movement into the cell interior, with actin polymerization occurring asymmetrically on the vesicle to drive movement. This latter situation is analogous to the movement of Listeria inside cells (reviewed in [75]).

Evidence for the fast movement of vesicles along actin cables that act as tracks originally came from studies of endocytic vesicles containing the lypophilic dye FM4-64. Uptake of the dye and subsequent movement of these vesicles to endosomes was followed and could be observed to take place on actin cables [51]. Likewise, a study tracking fluorescently labelled α-factor derivatives revealed that vesicles and endosomes move towards each other along actin cables [76]. In terms of the mechanism of movement, yeast cells do not contain a type VI myosin, and endocytic vesicles, rather than being actively moved along actin cables, have been shown to move with them as they polymerize and elongate [51]. Genetic evidence has also revealed interactions between proteins involved in cable formation (e.g. formins) and endocytic adaptors, such as Sla2p [77]. While all these data indicate that vesicles can move along cables, often no cables appear to localize to the exact point of endocytosis, and fusion to endosomes appears to happen in the absence of any cables. This is particularly the case in the growing bud where there are fewer actin cables. Thus, it is possible that while cables can be used, they might not represent the only mechanism for vesicle propulsion in the cell.

More recent studies have indicated the importance of Las17 in driving an actin-dependent event that is important in post-scission vesicle movement. Indeed, Las17 coupled with a binding partner, Lsb6, appears to be important in the movement to endosomes [47]. Furthermore, deletion of the acidic domain of Las17, which would preclude binding to Arp2/3, causes a marked defect in this late stage of endocytosis [21]. The idea of Las17 driving vesicle movement through the recruitment of appropriate factors onto the vesicle membrane is attractive, but to date the majority of researchers have reported Las17 as remaining in the non-motile patch at the plasma membrane and, therefore, it is not in the appropriate place to drive such movement following scission. One possibility is that the amount of Las17 that relocalizes to the vesicle side of the scission machinery is too small to observe with current live cell approaches. In support of this, cells carrying GFP-tagged Las17 as the sole source of Las17 in cells do not appear to undergo the same degree of post-scission movement as cells carrying another reporter [21]. This may suggest that the GFP tag interferes with the ability of Las17 to localize appropriately at this late stage of endocytosis, thus preventing its role in normal movement. Furthermore, in an ultrastructural study of the endocytic invagination, Las17 could be observed at the point of scission—100 nm away from the plasma membrane—providing strong evidence that it can be localized in an appropriate place to move with the vesicle after scission [34]. The data currently available, therefore, may favour a situation in which both vesicle-driven actin polymerization and an association with actin cables could be involved in post-scission movement to endosomes. The former might dominate in buds of cells, while the latter may be more common in larger mother cells that have more cables running close to the plasma membrane.

Lipids and the regulation of actin in endocytosis

Clearly, given that the endocytic complex is involved in invagination and scission of membrane, the nature of the lipids within the membrane is central to the overall mechanism. In particular, appropriate lipids must be present to allow both positive and negative curvature of the membrane. The tip of the invaginating endocytic pit exhibits positive curvature on its cytoplasmic side, while the region at which the membrane curves in, away from the

plane of the plasma membrane, will be negatively curved. The head group size and the charge of these head groups are both likely to play a role in enabling this curvature to take place. Other lipids that are known to play a role in endocytosis are the sphingolipids and ergosterol, both of which may be involved in stabilizing sites of endocytosis. It is beyond the scope of this review to describe all aspects of the role of lipids in endocytosis, and we have limited our discussion to recent work on the Arf3 protein, which has been shown to influence both PtdIns(4,5)P2 levels and actin organization.

Arf3 is the budding yeast homologue of Arf6 that has been shown to play a role in actin organization, endocytosis and the regulation of PtdIns(4,5)p2 levels in mammalian cells [78, 79]. Initial studies suggested that deletion of Arf3 does not have an endocytic phenotype. However, more recent data suggest that despite the lack of severe phenotypes, there are many strong lines of evidence to place Arf3 in an endocytic role. First, it localizes to the plasma membrane and interacts with a protein, Lsb5, which in turn interacts with Sla1 and Las17 [48, 49]. More recently, interactions have also been shown with ArfGEF and ArfGAP proteins. Of these, its guanine exchange factor (GEF) localizes to the plasma membrane in an Arf3-dependent manner [80, 81]. The ArfGAP, Gts1 shows extensive overlap in its localization with the endocytic machinery and interacts with many components, including Sla1, Pan1, YAP1801/2 and the clathrin light chain. These various data clearly link Arf3 with the endocytic machinery. Analysis of plasma membrane PtdIns(4,5)P2 levels indicate that Arf3 can serve to increase production of this lipid, presumably via the only phosphatidylinositol 4 phosphate 5 kinase in yeast, Mss4 [81]. The production of PtdIns(4,5)P2 is known to be critical for endocytosis as a site for binding the ANTH domain protein Sla2 [32]. However, some other interactions may be more directly important for regulation of actin. A recent report demonstrated that overexpression of Arf3 can rescue patch defects caused by deletion of the yeast WASP and WIP homologues, Las17 and Vrp1, respectively. In addition, overexpression of Arf3 rescues actin cable defects of yeast profilin mutants [82]. These results could be explained by the data demonstrating the effects of Arf3 on PtdIns(4,5)P2 levels. A regulatory interaction between profilin and PtdIns(4,5)P2 is well documented, and profilin has also been suggested to bind to Vrp1, which in turn regulates Las17p. Other actin-binding proteins in yeast involved in endocytosis, such as capping protein and twinfilin, also appear to be regulated by PtdIns(4,5)P2 in the membrane. Thus, a large increase in PtdIns(4,5)P2 in cells overexpressing Arf3 (or deleted for its GAP, Gts1) may be expected to have substantial effects on factors regulating and organizing actin, which could result in the effects reported by Lambert and colleagues. One interesting follow-up to these experiments will be to determine whether the endocytic patches that are rescued in the WASP and WIP deletion strains by Arf3 overexpression not only appear in the wild type but also behave in a normal way in live cell assays of endocytic reporters.

Ultrastructural studies of the invaginated pit

While electron microscopy has been a central methodology in generating our understanding of clathrin-mediated endocytosis in mammalian cells, there have been few studies in yeast that have used this approach as a primary study tool. The reason for this lies in problems with the density of the cell wall and yeast cytoplasm, which make the fixation and preservation of the cell ultrastructure relatively difficult.

Our first ultrastructural view of the invaginated patch was published some 15 years ago [67]. Images in this work showed pronounced invaginations at the plasma membrane and also localization of actin, cofilin and Abp1 to these structures. At this stage, it was not known that they were intermediates of endocytosis, and there was a suggestion for a role in cell-wall growth or osmotic regulation. However, later studies linked similar invaginations with defective endocytosis [9, 23].

The importance for an ultrastructural understanding of endocytosis has come to the fore recently with a need to understand the nature of the spots that are visualized in the many studies on live cells. There has been the recent publication of an elegant benchmark study in the field in the form of a careful analysis of the immunolocalization of a number of endocytic proteins at the invaginating pit [34]. By analysing plasma membrane invaginations, Idrissi et al. demonstrated that primary endocytic profiles in yeast are tubular invaginations of approximately 50 nm and up to 180 nm in length, with deeper pits corresponding to more mature sites of endocytosis [34]. Their experiments showed that (1) the endocytic coat components in these vesicles localize to the tip, (2) clathrin itself appears to form a flattened patch at the distal tip, (3) a slightly bent coated pit is formed before the initiation of the slow inward movement, (4) actin forms a loose cloud round the endocytic pit and (5) Myo5 accumulates at the membrane proximal side of the invagination. These observations are in striking agreement with the current model generated from live cell imaging approaches [14, 34]. As mentioned above, an unexpected finding was that while Myo5 was located to the plasma membrane side of the invagination, Las17p was distributed along the invaginating tubule as far as the central zone. Full-length Las17-GFP has not been visualized away from the plasma membrane—possibly indicating a function impaired by the GFP tag. These findings

strongly support the wider use of further ultrastructural studies to complement the live cell imaging approaches.

Actin in yeast endocytosis—summary and remaining questions

While it may appear that recent real-time fluorescence and electron microscopy studies have identified all of the major players and elucidated the endocytic process save for a few esoteric details, this is not the case. Indeed, while we now have a conceptual framework, many details of the mechanism remain unanswered. For example, we still know little of the molecular interactions that trigger the actual invagination step of endocytosis, or whether endocytic events for distinct cargoes are identical. It seems unlikely that all of the cargo binding signals have been identified. Later stages of the process are relatively undefined, and we do not know which factors limit the inward growth of the invagination and thereby elicit scission at the appropriate site. A final consideration is the state of the membrane itself. While it appears that scission occurs about half way down the invagination, what then happens to the excess membrane that remains? Does it simply return to the plane of the membrane? Is this same site reused? Ongoing and future studies using a mix of live cell and electron microscopy should over the next few years lead to a clearer elucidation of these questions.

Actin and endocytosis in mammalian cells

The conclusion from the yeast studies is that actin functions at distinct and sequential steps in endocytosis. Notably, actin polymerization drives invagination, possibly scission, and then post-scission movement of the newly formed vesicle. So, what is the evidence for actin in these roles, or indeed others, in higher eukaryotes?

Early observations connecting actin and its binding partners to components of the endocytic machinery [83] coupled with the observation of actin filaments in close proximity to sites of endocytosis at the synapse [84] suggested that actin might also be important for endocytosis in mammalian cells. However, treatment of cells with drugs, such as latrunculin or cytochalasin, that disrupt the actin cytoskeleton caused variable effects on endocytosis. This led to a model whereby actin may facilitate endocytosis depending on cell context, rather than having a mandatory role in clathrin-mediated endocytosis [85]. Recent studies, based largely on live-cell imaging, has provided compelling evidence for a role for actin in endocytosis, and progress has been made to elucidate the mechanistic basis of actin involvement (Fig. 3).

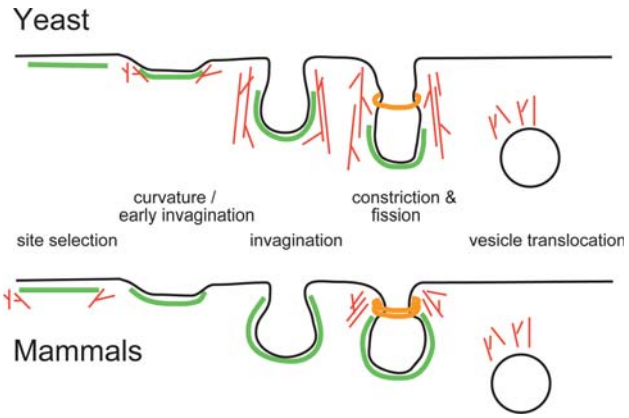

Fig. 3 Schematic comparing actin function in mammalian and yeast endocytosis. Actin has been shown to play roles at multiple distinct stages in endocytosis as shown. In mammalian cells, the exact stages of actin's involvement may vary according to cargo and cell type, although a role in scission and vesicle translocation appear to be widespread. In yeast, actin is required for most stages, with the possible exception of site selection, as it is well documented that actin arrives after the site has been selected

Localization of actin to clathrin-coated pits

Among the strongest evidence for a role for actin in mammalian endocytosis is that provided by studies which combined epifluorescence with total internal reflection fluorescence microscopy (TIRFM), thereby allowing the internalization of individual coated pits to be followed in living cells [86–88]. Using cells expressing a number of markers, including a fluorescently tagged form of clathrin light chain (DsRed CLC) and a GFP-tagged component of the Arp2/3 actin nucleation complex [86], a transient colocalization of Arp2/3 with clathrin just prior to internalization was revealed. The overall timing of assembly and disassembly of the spots was remarkably similar to the timing of endocytic patch formation in yeast. Additionally, a significant number of yeast actin binding proteins have mammalian homologues that interact with, and are required for the function of the endocytic machinery, providing further support for a role in mammalian endocytosis (Table 1).

As discussed above, actin functions in yeast during invagination, scission and the inward movement of vesicles. In mammalian cells, evidence points to a role for actin during invagination and scission and also during the initial formation of clathrin-coated pits [90]. Stage-specific assays that distinguished between invaginated coated pits and coated vesicles [89] indicated that both latrunculin A- and jasplakinolide-treated cells showed a reduction in transferrin internalization at a stage prior to invagination [90]. This appeared to result from a block in the assembly of newly coated pits and also a reduction in the lateral movement of clathrin patches on the plasma membrane.

Other events, including invagination and scission, were also shown to be dependent on actin. Here we will discuss what is known of the mechanistic basis for the role of actin at different stages of clathrin-mediated endocytosis.

Coat assembly and invagination

The PCH/F-BAR protein family has been implicated in the dynamic interplay of actin and the endocytic machinery [91, 92]. The F-BAR domain of these proteins allows them to bind phosphoinositides and to bend and tubulate membranes, as shown in liposome-based assays. Several family members can bind dynamin and co-localize with dynamin and actin at the cell surface. Overexpression of the F-BAR proteins causes tubules to form from the plasma membrane, an effect that is accentuated by treatment of cells with latrunculin-A [92]. Co-overexpression of wild-type but not mutant dynamin inhibits tubulation, leading to the suggestion that both dynamin and the rigidity of the actin cytoskeleton limit the ability of these proteins to tubulate membranes and, therefore, to co-operate in regulating the degree of invagination and constriction of clathrin-coated pits [91].

A recent advancement in studies of the F-BAR proteins has also provided intriguing mechanistic insight coupling membrane curvature and actin polymerization. Building on previous studies mentioned above [92], Takano et al. [93] have now shown that F-BAR proteins Toca-1 and FBP17 can both bind to membranes and also interact with the WASP-WIP complex through an SH3 domain interaction. Importantly, they also revealed that this interaction can induce WASP/WIP-mediated activation of actin polymerization in a manner dependent on membrane curvature. While this work was performed on liposomes in vitro, it clearly demonstrates a mechanism that could be extrapolated to the endocytic process for coupling actin polymerization to membranes in a manner dependent on the extent of curvature.

Interestingly, the use of electron microscopy revealed an accumulation of deeply invaginated structures in latrunculin-treated cells, suggesting that, unlike yeast, there is no absolute requirement for actin during invagination [90]. However, the possibility of differential requirements for actin in invagination, depending on cell type and context, is borne out by studies by Rocca et al. [94] who showed that the endocytosis of AMPA receptors in neurons requires the inhibitory action of a PDZ–BAR domain protein, PICK1, on Arp2/3-mediated actin polymerization. These studies implicate actin polymerization as a brake on uncontrolled membrane invagination in neurons that needs to be overcome to permit incorporation of AMPA receptors into invaginated clathrin-coated pits and their subsequent internalization. A mutant form of PICK1 that cannot interact with Arp2/3 has the expected dominant negative effect on endocytosis; this was overcome by treating cells with low concentrations of latrunculin-A. Interestingly, although PICK1 is necessary for AMPA receptor internalization in an heterologous system (Cos7 cells), latrunculin-A fails to rescue the effect of dominant negative PICK1, illustrating the diverse ways in which cells may mobilize the actin machinery during endocytosis.

Scission

Mammalian Abp1p (mAbp1), like its yeast orthologue Abp1, provides a potential link between the endocytic pathway and the actin cytoskeleton. Early studies showed that mAbp1 binds to dynamin via its SH3 domain and that the two proteins interact in vivo. In addition, overexpression of the SH3 domain of mAbp1 had a dominant negative effect on endocytosis [95]. Studies in mAbp1-knock-out mice revealed defects in the immune response as a result of impaired T-cell endocytosis [96]. Mice lacking mAbp1 also exhibit reduced rates of endocytosis, synaptic vesicle recycling and suffer from behavioural abnormalities. Analysis of mAbp1 knockdown cells revealed that the absence of mAbp1 affects events following vesicle scission and causes a significant reduction in the rate of synaptic vesicle repriming [97].

Huntingtin-interacting protein (HIP1R), the mammalian homologue of Sla2p, also appears to be involved in the function of actin during scission. HIP1R binds to clathrin light chains, cortactin and actin and additionally has an ANTH domain that can bind to PtdIns(4,5)P2 [98]. It was hypothesized to bridge endocytic vesicles to the actin cytoskeleton [99, 100]. HIP1R knockdown by siRNA in cells and fibroblasts from mice lacking the related HIP1 protein have reduced rates of vesicle formation [101]. In the absence of HIP1R, actin, dynamin and cortactin accumulate at sites of endocytosis, suggesting that HIP1R may also modulate turnover of actin at endocytic sites [101]. Recent studies have shown that binding to clathrin light chains substantially reduces the affinity of HIP1R for actin without affecting its binding to cortactin [102]. Cortactin is an activator of the Arp2/3 complex that is linked to endocytosis via its interactions with dynamin and its localization to coated pits [103]. Live cell imaging has revealed that HIP1R is recruited throughout coated pit formation, while cortactin appears as a sharp burst just before the onset of scission [104]. The interaction of HIP1R with cortactin appears to be important to its role in regulating actin assembly at coated pits since the accumulation of actin at endocytic sites in the absence of HIP1R can be overcome by cortactin depletion [104]. Cortactin in complex with HIP1R is unable to bind dynamin-coated liposomes or indeed to activate Arp2/3

and that HIP1R/cortactin acts as an actin capping protein; this provides a potential mechanism for the segregation of cortactin function in actin nucleation and capping [104]. A key issue for the future will be to understand the spatial and temporal regulation of cortactin interactions. Reversible phosphorylation is obviously one attractive and plausible regulatory mechanism, and it has already been shown that cortactin is a substrate for tyrosine phosphorylation by Src kinases and that tyrosine phosphorylation enhances the association of contraction with dynamin [105].

It is worth noting that in the cell lines analysed to date, cortactin is only present at a subset of coated pits—in contrast to HIP1R which is present at all pits [88, 104]. This strongly suggests that the negative regulation of actin assembly at, or following scission, may be mediated via multiple pathways. Other potential candidates to mediate Arp2/3 activation include N-WASP (homologue of yeast Las17), which is also recruited to coated pits [106]. Depletion of N-WASP by RNAi led to a reduction in the uptake of the epidermal growth factor receptor (EGFR) [107, 108]. Other well-characterized endocytic proteins that have been implicated in the recruitment of N-WASP to the clathrin-coated pits include syndapin, endophilin, intersectin-1 and SNX9 [109–111, 133].

A role for actin in scission is supported from the work of Kochubey et al. [112] in *Drosophila* hemocytes in which clathrin-enhanced GFP was expressed in mutants (*wasp* and *shibire*) defective in actin and dynamin function. These researchers demonstrated that actin plays a critical role in clathrin-mediated endocytosis in these cells and that actin and dynamin act at the same stage of endocytosis (scission) but independently of one another.

Recent studies have demonstrated the importance of actin as a regulator of scission in a developmental context. During morphogenesis in *Drosophila*, the embryo undergoes several cell cycles without cytokinesis to give rise to a syncytial embryo. During cellularization, the process whereby a syncytial embryo becomes divided into individual cells, Nullo, a regulator of actin dynamics, regulates scission to ensure successful furrow ingression [113].

Further links between actin and endocytosis

Identification of a conserved motif that mediates an interaction between members of the CIN85/CD2AP protein family and actin capping protein highlights another link between actin and endocytic proteins [114]. CIN85 and CD2AP are the most likely mammalian orthologues of Sla1p, although their primary roles may be cell-type dependent as they appear to be required for specific cellular events [115]. Like Sla1, CD2AP and CIN85 interact with multiple components of the endocytic machinery and actin cytoskeleton (CIN85: endophilin, synaptojanin, HIP1R [116]; CD2AP: rab4, AP2, cortactin and actin [117]), and disruption of this interaction leads to reduced downregulation of the T-cell receptor in vivo [118]. One possibility is that this protein family is involved in the uptake of specific cargo(es) that may mobilize particular components of the actin machinery. CIN85 specifically mediates the internalization of activated EGFR and c-Met receptors via interactions with the endocytic protein endophilin [119, 120], and CD2AP links EGFRs to cortactin [121]. It will be of interest to investigate if targeted disruption of the conserved motif linking CIN85/CD2AP to capping protein affects endocytosis of these receptors in vivo and also to determine whether this interaction is important early or late in coated vesicle formation.

Actin involvement in other endocytic events

In addition to clathrin-mediated endocytosis, there are other routes of entry into mammalian cells. It has been known for many years that phagocytosis, the engulfment of large particles, such as bacteria and cellular debris, and macropinocytosis, a mechanistically related process that results in trapping of large amounts of extracellular fluid following fusion of membrane ruffles, are both actin-dependent processes. More recently, it has been shown that there are alternative pathways to clathrin for the uptake of subpopulations of membrane proteins. Specifically, the GEEC [glycosylphosphatidylinositol (GPI)-anchored protein (AP)-enriched early endosome compartment] pathway appears to be specialized for the uptake of GPI-linked proteins in a process that requires the Rho family GTPase, Cdc42 [122], and is hijacked by the pathogen, *Helicobacter pylori* [123]. This pathway is also actin-dependent [124]. Sorting nexin9 (SNX9) has been implicated in the regulation of this pathway [111] as well as in clathrin-mediated endocytosis [125]. SNX9 was originally identified as a dynamin binding partner and, consistent with this identification, it localizes to clathrin-coated pits and participates in clathrin-mediated uptake [126]. Recent studies have shown that SNX9 is also involved in regulating actin dynamics on the GEEC pathway. In vitro, SNX9 stimulates N-WASP/Arp2/3-dependent actin assembly in a PtdIns(4,5)P2-dependent manner. It will be of interest to determine whether SNX9 performs similar functions in both clathrin-mediated and GEEC endocytosis [110].

A role for actin polymerization as a negative regulator of endosomal recycling of EGFR has been recently demonstrated in NRK cells [127] although actin appears to act to positively regulate synaptic growth via endosomal recycling in *Drosophila* [128] again indicating cell-specific regulatory mechanisms.

Perspectives

Advances in live cell imaging techniques and the development of a number of different fluorescent spectral variants to tag proteins have been key to our rapidly growing understanding of the endocytic process. It now seems likely that actin is involved in multiple stages of endocytosis in both yeast and higher eukaryotes. While these findings present a significant step forward in our overall understanding of endocytosis, they also present us with many new questions. At each stage of endocytosis, actin organization and dynamics may be governed by distinct signals and interactions with different subsets of proteins. At early stages of endocytosis, actin appears to play a role in site selection in some mammalian cells, or possibly in stabilizing the site in yeast. At later stages, actin organization into bundles is absolutely required for invagination in yeast, while this does not appear to be the case in the mammalian cells studied so far. Both yeast and higher eukaryotes appear to require actin for vesicle scission and vesicle movement away from the plasma membrane. In addition to these stage-specific functions of actin, its role might also vary according to cargo and cell type. The complexity of the protein interactions at each stage of endocytosis coupled with the range of cell types and assays used explain why reports have not always been consistent in ascribing a clear role for actin in endocytosis.

Thus, while recently developed technologies have allowed us to generate a conceptual framework for a mechanistic understanding of endocytosis, these advances have also revealed new levels of complexity in the process. Future studies to tackle these new questions will undoubtedly benefit from the continued use of model organisms and the ongoing development of even higher resolution live cell imaging capabilities.

Acknowledgments We would like to thank Soheil Aghamohammadzadeh and Alison Motley for critical reading of the manuscript. AR is supported by a BBSRC studentship, work in the ES lab is supported by a programme grant from the MRC (G0300452). KRA is a senior MRC non-clinical fellow (G0601600).

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