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Actin dynamics and endocytosis in yeast and mammals

Brian J. Galletta[*], **Olivia L. Mooren**[*], and **John A. Cooper**
Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, MO 63110

Abstract

Tight regulation of the actin cytoskeleton is critical for many cell functions, including various forms of cellular uptake. Clathrin-mediated endocytosis (CME) is one of the main methods of uptake in many cell types. An intact and properly regulated actin cytoskeleton is required for CME in *Saccharomyces cerevisiae*. Yeast CME requires the proper regulation of actin polymerization, filament cross-linking, and filament disassembly. Recent studies also point to a role for F-BAR and BAR domain containing proteins in linking the processes of generating and sensing plasma membrane curvature with those regulating the actin cytoskeleton. Many of these same proteins are conserved in mammalian CME. However, until recently the requirement for actin in mammalian CME was less clear. Several recent studies in mammalian cells provide new support for an actin requirement in the invagination and late stages of CME. This review focuses on the regulation of the actin cytoskeleton during CME in yeast and the emerging evidence for a role for actin during mammalian CME.

Introduction

The actin cytoskeleton is utilized in a variety of cellular processes including uptake of nutrients, down-regulation of signaling, motility, and cytokinesis. It is tightly regulated by accessory proteins that promote nucleation and subsequent growth of new actin filaments, the stabilization of an actin network, and the disassembly and turnover of actin filaments, which generates a new G-actin pool ready for another round of polymerization. Regulation of the actin cytoskeleton is important for endocytosis, and this review will focus on the contributions of the actin cytoskeleton to clathrin-mediated endocytosis (CME).

The role of the actin cytoskeleton in endocytosis in budding yeast

Endocytosis and actin assembly in yeast have been linked for some time. Mutants in actin related proteins have been isolated in screens for endocytic mutants, mutations in actin binding protein genes cause endocytic defects, and endocytic and actin assembly proteins colocalize. The definitive connection between these two systems came with the advent of sophisticated live cell imaging in yeast, which demonstrated coordinated assembly and disassembly of endocytic and actin-associated proteins at actin patches [1]. In addition, actin patch components were shown to colocalize with FM4-64 (a lipid dye) and fluorescently labeled alpha-factor (a ligand for a membrane receptor) [2,3].

Correspondence to: John Cooper, Campus Box 8228, 660 S. Euclid Ave., St Louis, MO 63110, jcooper@wustl.edu, phone: 314-362-3964.
*These authors contributed equally to this work

A stereotypical series of events takes place at sites of endocytosis. First, endocytic proteins and adaptors, including clathrin, Sla1, Sla2 and End3 appear at the actin patch, followed by regulators of the Arp2/3 complex, a nucleator for actin polymerization. These proteins remain relatively stationary near the cell cortex, presumably attached firmly to the plasma membrane. Near the end of this phase, the first signs of actin assembly appear, as actin-binding proteins, including Arp2/3, Abp1, fimbrin/Sac6, CP and others, appear. Next the majority of endocytic and actin binding proteins move a short distance away from the cell cortex, likely representing the invagination of the membrane to form an endocytic tubule. The fission of the vesicle from the tubule presumably takes place at the end of this phase. The endocytic proteins then leave, but the actin-binding proteins continue to move into the cytoplasm with the vesicle (Reviewed in [4–7]. Current research is focused on understanding the molecular details of how actin assembly at these sites is properly regulated; how actin assembly at these sites is coordinated with the rest of the endocytic machinery; and how the force generated by this actin network is used to drive invagination, scission and subsequent motility of the endocytic vesicle.

Regulation of actin assembly

Actin assembly is required to drive the inward movement of actin patches off the cortex [1, 8]. The actin at these sites is composed of a network of branched actin filaments [9] and the dendritic nucleation model for Arp2/3-based actin assembly may be a good model for how actin assembles at these sites. The major proteins involved in the dendritic nucleation model are all localized to these sites of endocytosis, including Arp2/3, capping protein (CP), and cofilin, and these proteins are all required for normal endocytosis [8,10,11]. The Arp2/3 complex appears to be the primary nucleator of actin at patches, and conditional loss of Arp2/3 function leads to significant defects in patch behavior [10]. The acute loss of formins, another major class of actin nucleator in yeast, does not result in a loss of actin patch assembly or proper endocytic motility [8].

The nucleation of actin filaments is a critical step in the formation of proper actin networks. In mammalian systems, the nucleation activity of Arp2/3 requires binding to a Nucleation Promoting Factor (NPF) [12]. In contrast, the yeast Arp2/3 complex is relatively active without a NPF [13], so simply localizing Arp2/3 properly may be sufficient to regulate its activity. In yeast there are five NPFs that localize to the actin patch [14]. Interestingly, while disruption of the Arp2/3 binding regions of these proteins can disrupt aspects of endocytosis, in all mutants examined to date, actin still accumulates at sites of endocytosis [15,16] (Galletta, BJ and Cooper, JA, unpublished observations). This may suggest that the major role of the NPFs may not be to induce Arp2/3 to nucleate filaments. A more detailed study of the precise effects of these NPF mutants will be required to confirm this hypothesis and understand the exact role of Arp2/3 binding by these NPFs. In addition, some mutations of Arp2/3 itself, which dramatically reduce the *in vitro* nucleation activity of Arp2/3, do not have a major effect on the accumulation of actin *in vivo* [17]. Thus, Arp2/3 may be important for more than simply nucleating new branched filaments during endocytosis in yeast and this raises the question of what is nucleating the filaments in actin patches [17].

In the cell, actin patch filament networks are very dynamic, so that regulation of their disassembly is likely to be important. Three proteins, cofilin, Aip1 and coronin, which *in vitro* studies suggest work together to disassemble actin, are present in the patch [18–24] Consistent with a role in actin network turnover, all three of these proteins arrive to the patch after actin assembly has begun [25], and loss of function mutants in these proteins slows the turnover of actin in cells [11,19,26]. Severe loss of function mutations in cofilin cause an extended lifetime at the cell cortex by patches, but they do not affect the initial movement of invagination. After leaving the cell cortex, actin patches in cofilin mutants move further into the cytoplasm, consistent with a defect in disassembling actin networks [25,27].

Loss of coronin, like cofilin mutants, results in an increased time spent at the membrane prior to initiation of movement and an increase in the distance moved after scission, although to a much lesser degree than described for cofilin mutants [15,25]. *In vitro*, the activity of coronin can change depending on the nucleotide state of the actin filament. On ATP actin filaments, coronin can protect the actin from cofilin, but on ADP actin, coronin can synergize with cofilin to sever actin filaments and coronin may have some severing activity of its own [19]. Loss of Aip1 also results in an increased time spent at the membrane prior to patch movement, but the movement of patches appears normal [25]. Aip1 may be acting to help generate actin monomers from short actin oligomers generated by cofilin. In its absence, cells accumulate short actin oligomers that may anneal, minimizing the impact of its loss on actin dynamics [28]. It should be noted that the actin patch motility phenotypes of cells where coronin and/or Aip1 have been deleted are much less severe than what is seen in cofilin mutants [25]. This suggests that if coronin and aip1 are cooperating with cofilin, they make relatively minor contributions to normal cofilin function and actin patch movement *in vivo*.

Linkage between actin and membrane bending proteins

The interplay between the actin network and the proteins that bind to, and can bend, the plasma membrane, is emerging as an important area of study. BAR and F-BAR proteins may serve to bend the membrane, as well as to sense membrane curvature [29]. In yeast there are several BAR and F-BAR domain proteins in the actin patch, including Syp1, Bzz1, Rvs161, and Rvs167.

Syp1 is recruited very early in the lifetime of the patch, with similar timing as clathrin. Syp1 is almost completely gone from the patch when actin polymerization begins [30–32]. Syp1 can bind to and tubulate liposomes in vitro. [32]. Most interestingly, Syp1 can negatively regulate WASp/Las17-Arp2/3 mediated actin polymerization *in vitro*. Boettner and colleagues have suggested an intriguing model where Syp1's BAR domain assists in generating the initial curvature of the plasma membrane, while simultaneously inhibiting actin nucleation. As the curvature of the membrane changes as endocytosis proceeds, Syp1 is released allowing actin nucleation to proceed [31].

Another F-BAR domain containing protein, Bzz1, is recruited to the patch after Syp1, just prior to the initiation of actin polymerization [16]. Bzz1 has the opposite effect of stimulating WASp-based actin polymerization. The SH3 domains of Bzz1 interact directly with WASp/Las17, and they are sufficient to induce actin polymerization on beads when incubated with yeast cell extracts. This actin polymerization depends on Arp2/3, Las17, Vrp1, and type-I myosins [33, 34]. In *in vitro* polymerization assays Bzz1 can relieve the inhibition of Las17 activity by Sla1 [16]. Toca-1 and FBP17, the likely mammalian counterparts to Bzz1, can recruit the N-WASp/WIP complex to the membrane and activate WASp/WIP to initiate actin polymerization in a membrane curvature-dependent manner [35]. An appealing model is that Bzz1 senses the curvature induced by earlier F-BAR proteins, like Syp1, and starts polymerization on the already curved membrane.

The BAR domain containing amphiphysin proteins, Rvs161 and Rvs167, arrive after actin polymerization has begun, at approximately the time when membrane fission is thought to occur. Consistent with a defect in membrane scission, Rvs167 localizes to the sides of endocytic invaginations by immuno-EM and cells lacking Rvs161 or Rvs167 show frequent retractions of endocytic proteins following their inward movement [36,37]. Rvs167 can bind to WASp/Las17, so it too may regulate actin network assembly [38].

The role of the actin cytoskeleton in mammalian endocytosis

As described above, there is an absolute requirement for the actin cytoskeleton in *S. cerevisiae* clathrin-mediated endocytosis (CME). There is also evidence for a role for actin in CME in *Drosophila* [39]. In mammalian systems, the actin cytoskeleton is known to participate in various modes of cellular uptake from the environment, including phagocytosis, macropinocytosis, and caveolin-mediated endocytosis. However, the requirement for actin in mammalian CME is less clear.

In mammalian systems, there is strong biochemical evidence for interactions between many of the endocytic adaptors and the actin cytoskeleton, including several conserved from *S. cerevisiae*. Many proteins that link the endocytic machinery and the actin cytoskeleton potentially regulate actin assembly at endocytic sites. For example, Hip1R, the mammalian orthologue of yeast Sla2, binds clathrin, F-actin and cortactin to negatively regulate actin assembly [40]. Intersectin binds epsin (similar to Ent1 and Ent2 in yeast) and N-WASp, an activator of actin assembly [41]. Both cortactin and mAbp1 (yeast Abp1) can activate actin assembly and bind to F-actin and to dynamin, the mammalian GTPase involved in vesicle fission from the plasma membrane [42–44]. Other proteins link dynamin to N-WASp, such as SNX9/SNX18, Tuba and the F-BAR-domain proteins syndapin, Toca-1/CIP4, and formin-binding protein 17 (FBP17) [35,45–52]. Additionally, the BAR domain protein PICK1, binds to Arp2/3 complex and inhibits actin polymerization *in vitro* [53].

Certain cell biology experiments support a role for the actin cytoskeleton in mammalian CME. For example, actin is recruited to clathrin-coated pits prior to movement of the vesicle away from the plasma membrane. Actin also contributes to the shape of the endocytic pit and the lateral movement of clathrin-coated pits within the membrane [54,55]. However, experiments that treat cells with reagents to disrupt the actin cytoskeleton have resulted in conflicting results, depending on the cell types [55–57]. Several recent papers have attempted to reconcile these inconsistencies and to determine the role of the actin cytoskeleton in mammalian CME.

Kirchhausen and co-workers recently performed a careful analysis of CME events and reported two classes of CME structures, termed coated pits and coated plaques [57]. These two classes of clathrin-coated structures had distinct kinetic properties, with short-lived coated pits characterizing the typical clathrin endocytic vesicle, and longer-lived coated plaques occurring at the adherent surface of certain cell types. Dynamic actin and actin-binding proteins, Hip1R and cortactin, were necessary for the formation and invagination of the coated plaque population, but not for the coated pit population. The authors argue that the existence of these two populations of clathrin-coated structures accounts for the discrepancies reported for a critical role for the actin cytoskeleton and actin-binding proteins in CME.

Locally regulated cortical tension on the plasma membrane of mammalian cells may differentially affect clathrin-coated pit kinetics [58]. Using BSC1 cells, which do not form the coated plaques described above but only coated pits, three subpopulations of clathrin-coated pits (CCPs) were described: early and late abortive (short-lived), and productive (longer-lived) [57,59]. Within the productive subpopulation, there was still heterogeneity in the kinetics of internalization, suggesting additional factors regulating CME.

A recent paper by De Camilli and co-workers demonstrated the concerted actions of BAR-domain proteins and the actin cytoskeleton to invaginate and tubulate membranes associated with clathrin pits prior to scission [60]. Cells derived from dynamin 1 and 2 double conditional knockout mice resulted in significant impairment of CME and the growth of long tubulated clathrin-coated pits to which BAR-domain proteins, F-actin and actin binding proteins colocalized. The actin cytoskeleton was required to maintain the tubules. The authors suggest a model of CME in which clathrin recruits additional endocytic components, including BAR-

domain proteins and actin binding proteins. Subsequent coordinating actions of BAR-domain proteins and actin promote the invagination and tubulation of clathrin-coated pits until dynamin is recruited resulting in fission of the vesicle from the membrane.

The results from De Camilli and co-workers are intriguing because they demonstrate parallels between yeast and mammalian cell CME mechanisms, which some investigators have considered to be distinct. These parallels would indicate that the actin cytoskeleton plays a conserved critical role in the invagination of clathrin-coated pits, and perhaps at later stages, such as fission and movement of vesicles away from the membrane subsequent to fission.

Danuser and co-workers measured lifetimes of clathrin-coated pits of cells attached to patterned substrates [58]. The lifetimes of CCPs within regions of the cell that were attached to fibronectin were significantly longer than the lifetimes of CCPs measured outside of the region of attachment. Using reagents to reduce cortical tension by disrupting the actin cytoskeleton resulted in a reduction of the lifetimes of CCPs in the attached regions so that there was no measurable difference in the lifetimes of the CCPs within the two regions. Therefore, localized changes in cortical tension via actin cytoskeletal rearrangements may contribute to CME and may account for the heterogeneity of the kinetics reported previously.

Recent studies suggest that because yeast have a cell wall and increased turgor pressure compared to mammalian cells, yeast CME may require a stabilized actin network to provide sufficient force for membrane invagination [61]. This is supported by additional evidence that disruption of the cortical actin cytoskeleton in mammalian cells reduces membrane tension and low expression of BAR-domain proteins can artificially induce long membrane tubules when the actin cytoskeleton is compromised [62,63]. Therefore, regulation of the actin cytoskeleton may modulate membrane tension to facilitate invagination of clathrin-coated pits in both yeast and mammalian cells.

Conclusions

Models for how actin assembly drives invagination

While it is clear that a dynamic actin cytoskeleton is essential for endocytosis in yeast and evidence is mounting for a role for actin in mammalian systems, precisely how this actin network is used to help provide the force required for the invagination, scission and vesicle motility during endocytosis is unclear. In one model, the actin filaments are primarily nucleated from a ring of NPFs around the base of the endocytic invagination. The growing barbed ends of the filaments remain pointed towards the plasma membrane, resulting in a flow of actin filaments away from the cortex. These filaments are attached to the membrane and the flow of this network away from the plasma membrane helps extrude the invagination into the cytoplasm (Figure 1A). While the flow of actin away from the cortex is supported by observations of *sla2*Δ cells, these cells have very severe defects in endocytosis and the large, flowing actin comet tails seen in these cells may represent a structure found very early during endocytosis or even a structure specific to cells blocked for endocytosis in this way. Furthermore, this model requires that new actin subunits, Arp2/3 and actin binding proteins like capping protein would be incorporated at the membrane as invagination takes place. However, there is no evidence for any of these proteins remaining at the membrane or occupying the region between the endocytic particle and the membrane. All of the actin associated proteins move into the cytoplasm and away from the membrane.

An alternative model is similar to one proposed by Merrifield and colleagues for animal cells and another one recently elaborated by Suetsugu [64,65]. In this model (Figure 1B) actin is polymerized around the endocytic coat, which has already generated some degree of curvature in the membrane. The presence of curvature prior to actin polymerization is supported by EM

data [37]. Furthermore, as documented above, F-BAR domain-containing proteins may help direct actin polymerization to invaginations. The force from these filaments can squeeze the invagination and help push the endocytic membrane into the cytoplasm. In this model, the actin machinery remains directly adjacent to the endocytic machinery and no connection to the plasma membrane is required. The actin filaments are in position to help during scission and to drive motility after membrane scission, which requires a properly functioning dendritic actin network [8,15,25,66].

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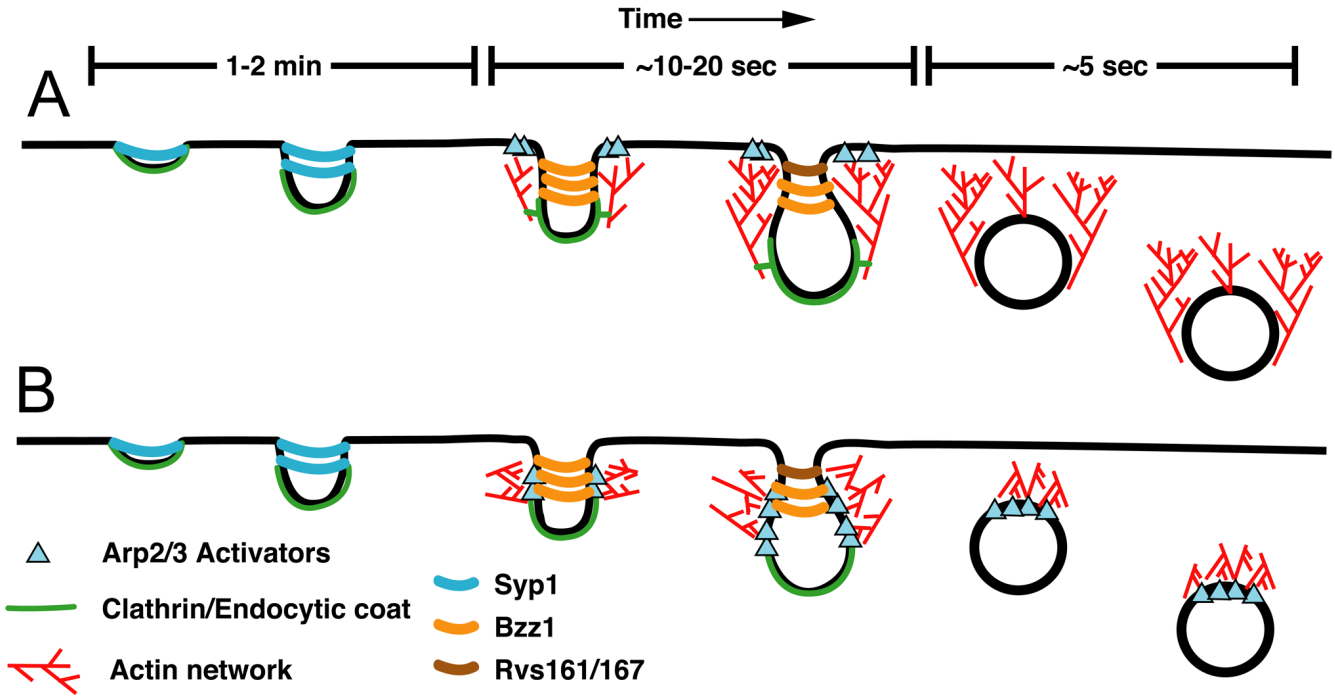

Figure 1.

Major models for actin assembly during endocytosis derived from the results of numerous works described and referenced herein. In both A and B the initial curvature of the membrane is generated by clathrin and additional endocytic proteins, such as F-BAR containing Syp1. Syp1 can inhibit WASp/Las17-Arp2/3 mediated actin assembly and may serve to keep actin polymerization inhibited during early steps of CME. As invagination proceeds, the changing membrane curvature may be sensed by other BAR domain containing proteins, for example Bzz1, which is recruited along with other endocytic proteins and regulators of Arp2/3. In model A, a ring of NPFs activate Arp2/3 which nucleates an actin network that flows away from the plasma membrane. Proteins of the endocytic coat link the membrane to this flowing network and this provides the force to invaginate further into the cell. The amphiphysin proteins, Rvs161 and Rvs167, drive membrane scission. In model B, actin is nucleated along the sides of the membrane tubule, perhaps in response to the loss of Syp1, and the recruitment of Bzz1, which can promote actin nucleation. The growing barbed ends of the actin push on the membrane tubule, squeezing it, driving elongation and assisting the amphiphysin proteins during membrane scission. In this model, the actin network is in a position to drive movement after the vesicle has been freed from the membrane. The best models for how actin polymerization is utilized during clathrin-mediated endocytosis in mammals are similar to the model presented in B [64,65]