

Coupling actin dynamics and membrane dynamics during endocytosis

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A convergence of cellular, genetic and biochemical studies supports the hypothesis that the actin cytoskeleton is coupled to endocytic processes, but the roles played by actin filaments during endocytosis are not yet clear. Recent studies have identified several proteins that may functionally link the endocytic machinery with actin filament dynamics. Three of these proteins, Abp1p, Pan1p and cortactin, are activators of actin assembly nucleated by the Arp2/3 complex, a key regulator of actin assembly *in vivo*. Two others, intersectin and syndapin, bind N-WASp, a potent activator of actin assembly via the Arp2/3 complex. All of these proteins also bind components of the endocytic machinery, and thus, could coordinately regulate actin assembly and trafficking events. Hip1R, an F-actin-binding protein that associates with clathrin-coated vesicles, may physically link endocytic vesicles to actin filaments. The GTPase dynamin is implicated in modulating actin filaments at specialized actin-rich structures of the cell cortex, suggesting that dynamin may regulate the organization of cortical actin filaments as well as regulate actin dynamics during endocytosis. Finally, myosin VI may generate actin-dependent forces for membrane invagination or vesicle movement during the early stages of endocytosis.

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Abbreviations

Arp2/3	actin-related protein 2/3
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Hip	Huntingtin interacting protein
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PRD	proline-rich domain
SH3	src-homology region 3
WASp	Wiskott–Aldrich syndrome protein

Introduction – are endocytosis and the actin cytoskeleton tightly coupled?

The most conclusive evidence in favor of tight coupling between the endocytic machinery and the actin cytoskeleton comes from genetic studies of budding yeast [1,2]; recent studies of yeast proteins are beginning to unravel the biochemical mechanisms upon which these genetic interactions are based. Conclusions pertaining to a connection between endocytosis and actin dynamics in mammalian cells are less strong than in yeast, and, in some cases, inconsistent. The identification, however, of several mammalian proteins that physically link proteins of the

actin cytoskeleton with those of the endocytic complex, together with observations of endosome and actin dynamics in live cells, has strengthened the notion that actin filaments participate during some steps of endocytosis. This review highlights these recent findings and discusses the mechanisms whereby actin filaments may facilitate endocytic traffic in mammalian cells.

In higher eukaryotic cells, much of the early evidence implicating actin in endocytic vesicle formation came from investigating the effects on endocytosis of reagents that disrupt the actin cytoskeleton. Conclusions about the requirement of actin assembly for endocytic uptake varied depending on which cells and growth conditions were used, which plasma membrane surface (apical or basolateral) was considered, which actin-disrupting agent was used, and which types of endocytosis assays were used to assess internalization [3–16]. Although much evidence supported a role for actin filament involvement during endocytosis in mammalian cells, this was not the case under all conditions. To revisit this question, a comprehensive study of the effects of three actin-perturbing drugs on the constitutive uptake of transferrin into constricted coated pits was carried out [17*]; recently, these published findings were extended to include effects of the drugs on coated vesicle formation (M Fujimoto, S Schmid, personal communication). Together, these experiments corroborated the conclusion that actin filaments facilitated constitutive receptor-mediated endocytosis under some conditions, but disruption of actin filaments did not universally perturb coated-vesicle formation. A role for actin filaments in events downstream of vesicle formation, such as uncoating, movement of newly formed vesicles through the cortex and into the deeper cytoplasm, or in recycling and sorting through endosomal compartments, was not ruled out [8].

Functions of the actin cytoskeleton during endocytic processes

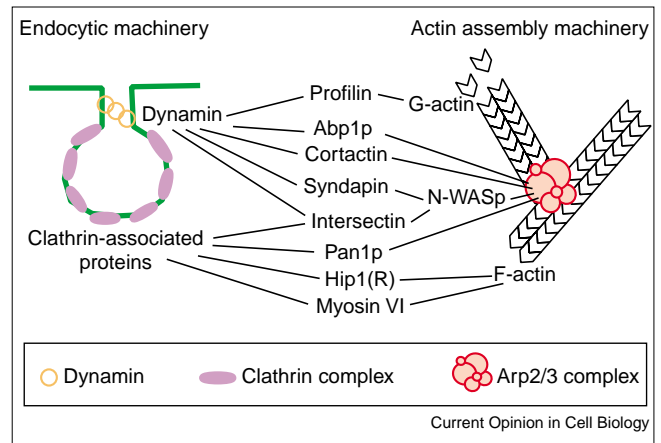
Actin filaments may play multiple roles during endocytosis. Actin filament assembly could generate mechanical forces to induce membrane invaginations, pinch off deep invaginations to create vesicles, or propel newly formed vesicles away from the plasma membrane. Myosin-dependent forces that require actin filaments could accomplish similar activities. Interactions of components of the endocytic machinery with actin filaments could initiate the assembly of the endocytic machinery or anchor it at nascent internalization sites. Local disassembly of actin filaments of the cell cortex near internalization sites could promote formation of nascent pits or facilitate passage of newly formed vesicles into the deeper cytoplasm by clearing the way for vesicle diffusion or interactions with microtubules. Several excellent discussions of proposed functions and the experimental data for actin filaments in endocytosis are available [18,19].

Rocketing endocytic vesicle movement about the cell is powered by actin assembly forming an F-actin 'tail'; observations of these rocketing endocytic vesicles are dramatic evidence that actin assembly can contribute to moving endocytic vesicles [20,21*,22,23]. Pinosomes induced by osmotic shock were observed to form at, and move away from, the plasma membrane of RBL cells via actin tails resembling those assembled by *Listeria* and other bacterial and viral pathogens [23]. It is not yet clear whether the actin polymerization associated with moving pinosomes is coupled to pinosome budding from the plasma membrane. Two factors that may regulate pinosome formation and motility are annexin 2, a Ca²⁺-binding and F-actin-binding protein, and ARF6, a small GTPase. Annexin 2 labeled with green fluorescent protein (GFP) was found to be a component of the F-actin tails formed on moving pinosomes in RBL cells, and more importantly, appeared to play a functional role in inducing rocketing pinosomes [24*]. Overexpression of a dominant-negative annexin 2 mutant abolished pinosome motility but had no effect on actin-tail formation and motility of *Listeria*. Thus, annexin 2 may control actin assembly at endosome membranes. Expression in fibroblasts of an active mutant form of ARF6 (ARF6[Q67L]) that keeps ARF6 in its GTP-bound form increased pinocytic uptake and induced rocketing pinosomes in fibroblasts [25*].

Identification of proteins that bridge the endocytic and actin machines

The identification of proteins that link actin filaments, particularly the stimulation of new actin polymerization, with proteins of the endocytic machinery is beginning to shed light on the mechanisms that couple actin filaments and endocytosis (Figure 1). Three such proteins, Abp1p and Pan1p of *Saccharomyces cerevisiae* and cortactin of mammalian cells are direct activators of actin filament assembly. The Arp2/3 complex is a key regulator of actin polymerization *in vivo* that promotes filament assembly through enhanced nucleation of actin subunits [26–28]. The complex is required for endocytosis in yeast [29] and participates in pinocytosis and phagocytosis in mammalian cells [30]. The Arp2/3 complex is also implicated in endocytosis in mammalian cells because T cells from WASp-knockout mice exhibit reduced levels of endocytosis [31]; WASp family proteins are potent activators of actin assembly via the Arp2/3 complex. Syndapins and intersectin indirectly link actin polymerization and endocytic proteins via interactions with WASp family proteins. Thus, given the multiple routes by which actin polymerization may be stimulated in association with endocytic proteins, it is tempting to speculate that actin assembly provides a critical, if not required, function for endocytosis. However, the challenge will be to determine whether *de novo* actin assembly provides a specific function for endocytosis or whether the actin assembly induced by these proteins provides other functions.

Figure 1



Summary of the protein–protein interactions identified as potential links between components of the endocytic and actin cytoskeletal machines. Black lines joining individual proteins indicate specific binding interactions for those components. The interactions were identified using a variety of techniques, including direct binding studies, immunoprecipitation and blot overlay experiments. The functional significance for the endocytic process of many of these interactions is not yet known; however, two types of functional links are apparent. First, activation of actin assembly by the Arp2/3 complex could occur via direct binding of activators (i.e. Abp1p, cortactin, Pan1p), or via activation of N-WASp, which may be recruited to nascent clathrin-coated pits by scaffolding proteins (i.e. syndapin and intersectin). Second, interactions of endocytic proteins with F-actin (Hip1[R] and myosin VI) and G-actin (profilin) may tether the machineries together and modulate the dynamics of each.

Cortactin, Abp1p and Pan1p

Cortactin, Abp1p and Pan1p, like the WASP family proteins, bind to the Arp2/3 complex and activate its actin nucleation activity [32*–35*]. Each of these proteins also interacts with endocytic proteins, but the significance of these interactions for endocytosis is not well understood. Cortactin and the Arp2/3 complex are associated with motile endosomal vesicles in the cytoplasm [36], suggesting that cortactin may promote the actin assembly that propels endosomes. Cortactin and mouse Abp1 (mAbp1) were recently shown to bind dynamin, a key GTPase involved in endocytic vesicle formation [37,38], via interactions of their SH3 domains and the proline-rich domain (PRD) of dynamin [39*,40*]. Cortactin and mAbp1 both localize with dynamin in growth factor treated fibroblasts, a condition that stimulates cortical actin dynamics. Cortactin and dynamin are enriched with F-actin at the leading edge of the lamellipodia and are also enriched at punctate spots within the lamella [39*]. In resting cells, mAbp1 is not detected with F-actin or endocytic compartments, but its association with F-actin at the leading edge, and with dynamin at punctate spots throughout the lamella is dependent on growth factor receptor activation [40*]. Proteins of the endocytic machinery, including AP2, eps15 and Hip1R, also are located at dynamin-containing spots throughout the lamella, suggesting that mAbp1 is enriched at sites of endocytosis in

stimulated cells. In addition, overexpression of the mAbp1 SH3 domain in Cos-7 cells disturbed receptor-mediated endocytosis [40^{*}]. Unlike yeast Abp1, mAbp1 lacks the canonical acidic sequences associated with binding to the Arp2/3 complex, and has not yet been shown to stimulate the actin nucleation activity of the Arp2/3 complex. mAbp1, however, might interact and activate actin nucleation by the Arp2/3 complex via a novel mechanism. Mouse Abp1, like cortactin, binds F-actin and could thereby link dynamin to cortical actin filaments at the necks of nascent endocytic vesicles.

Pan1p of budding yeast is essential for the internalization step of endocytosis [41] and interacts with four clathrin-binding proteins. Its mammalian homolog eps15 is associated with clathrin-coated pits through interactions with the clathrin assembly complex AP-2 and epsin [42,43]. Pan1p also binds and activates the actin nucleation activity of the Arp2/3 complex [35^{*}] making Pan1p the first yeast protein identified that could directly link actin dynamics with endocytic processes.

Huntingtin interacting proteins (Hip)

Hip1 and Hip1R are mammalian orthologs of yeast Sla2p, which is a component of the yeast actin cytoskeleton and is required for the internalization step of endocytosis [44]. Hip1 and Hip1R bind F-actin *in vitro* and are associated with clathrin-coated vesicles [45^{*},46,47]. The interaction of Hip1 with endocytic compartments is likely to be mediated by direct binding to clathrin and α -adaptin A and C [45^{*}]. In live cells, GFP-tagged Hip1R exhibits a dynamic distribution, similar to that observed for GFP-clathrin [48]. Hip1R binds clathrin cages *in vitro* via interactions that depend on clathrin light chains [47]. The punctate structure of the cortex that contains clathrin and Hip1R appear and disappear simultaneously, presumably reflecting the assembly of coated pits and their subsequent uncoating as they 'pinch off' the plasma membrane [47,48]. Hip1/Hip1R may function to anchor newly forming coated pits to the cortical cytoskeleton.

PACSIN/syndapin and intersectin

Protein scaffolds that interact with several different proteins through assorted protein-protein interaction domains play important roles in endocytosis. PACSIN/syndapin and intersectin-1, the neuronal isoform of intersectin, may provide indirect links between actin assembly and endocytic processes through interactions with endocytic proteins and N-WASp [49,50^{*}]. WASp family proteins are the most potent and best-characterized activators of actin nucleation by the Arp2/3 complex [51]. N-WASp is also located at the interface of endocytic vesicles and F-actin tails [21^{*},25^{*}]. PACSIN/syndapin and intersectin each bind several proteins involved in endocytosis. PACSIN/syndapin binds dynamin, synaptojanin and synapsin, and appears to participate in endocytosis since overexpression of the SH3 domains of syndapin-I and -II inhibits receptor-mediated uptake [49,52^{*}]. Overexpression of full-length syndapin in

HeLa cells induces the formation of filopodia apparently via Arp2/3 complex-mediated actin polymerization [52^{*}]. PACSIN/syndapin also interacts via its SH3 domain with mSos, a guanine nucleotide exchange factor (GEF) for Ras and Rac [53], suggesting another route by which interactions of molecular scaffolding proteins with specific binding partners may coordinate signals to the actin cytoskeleton and to the endocytic machinery. The regulation of this complex network of interactions mediated by the PACSIN/syndapin SH3 domain must be exquisitely controlled in order to coordinate membrane and cytoskeletal activities.

Intersectin is a scaffolding protein that regulates the formation of clathrin-coated vesicles [54]. The neuronal isoform of intersectin, intersectin-1, may also promote Arp2/3 complex-dependent actin assembly through activation of N-WASp and Cdc42 [50^{*}]. GTP-bound Cdc42 together with phosphatidylinositol 4,5-bisphosphate (PIP₂) are co-activators of N-WASp, enabling it to stimulate actin nucleation by the Arp2/3 complex [55]. Intersectin-1 has a Dbl homology (DH) domain at its carboxyl terminus that exhibits GEF activity for Cdc42; the GEF activity on cdc42 is enhanced by binding of N-WASp [50^{*}]. Thus, intersectin-1 can activate Cdc42 and binds a Cdc42 effector protein, N-WASp, providing a novel mechanism for stimulating actin assembly. Filopodia, actin-containing cortical structures formed as a result of Cdc42 activation, were induced in cells expressing the intersectin-1 DH domain [50^{*}].

Although PACSIN/syndapins and intersectin-1 affect both cytoskeletal and membrane trafficking events, it is not yet known whether these scaffolding proteins synergistically orchestrate actin and membrane dynamics or whether they regulate actin and membrane dynamics to affect separate pathways.

Myosins and endocytosis

In yeast, the class I myosin Myo5p is required for receptor-mediated endocytosis [56]. The first evidence that myosin was involved in the early stages of clathrin-mediated endocytosis in mammalian cells implicated myosin VI in the process. Myosin VI is unusual in that it is the only known myosin to move towards the pointed ends of actin filaments [57]. One isoform of myosin VI, a spliced variant that contains an insert within the carboxy-terminal globular tail and that is expressed predominately in polarized cells, appears to be involved in clathrin-dependent processes [58^{*}]. This variant is targeted to cortical sites where it is co-localized with clathrin-coated pits and vesicles; myosin VI also was associated with a protein complex containing clathrin and adaptor protein AP-2. Furthermore, overexpression of the myosin VI tail domain reduced transferrin uptake in cells. Thus, myosin VI, together with the actin filaments with which it interacts, may function to deform and invaginate the plasma membrane during the early stages of coated-pit formation. Alternatively, myosin VI is also a good candidate

for moving endocytic vesicles through the dense actin filament network found in the terminal web domain at the apical surface of polarized cells that may otherwise be difficult to negotiate.

A role for dynamin in regulating actin dynamics

The GTPase dynamin, a key component of the endocytic machinery, has been implicated in regulating cortical actin dynamics, a function that may be independent of its participation in endocytosis. Dynamin isoforms are enriched in several actin-rich structures of the cell cortex. Dynamin is recruited to cortical membrane ruffles induced in growth factor-treated fibroblasts [39*,59] and to actin-rich phagocytic cups in macrophages [60]. A role for dynamin 2 in modulating cell shape was suggested by the finding that fibroblast morphology was altered in cells expressing a truncated form of dynamin 2 lacking the PRD domain [39*]. Dynamin 2 also is enriched at podosomes, which are dynamic, actin-rich structures at the ventral plasma membrane where cells contact the substratum [61*]. The function of podosomes is unclear, but they may be specialized adhesive structures involved in cell migration. Expression of a mutant form of dynamin 2 with decreased GTP-binding activity disrupted actin assembly at podosomes [61*], providing a clue that the GTPase activity of dynamin may regulate actin dynamics. Expression of a similar mutant of dynamin 2 in macrophages did not appear to disrupt actin assembly required for formation of phagocytic cups, but phagocytosis was blocked at a later step, resulting in failure to completely internalize particles [60]. Finally, antisense depletion of dynamin 1 in cultured hippocampal neurons impaired the formation of neurites [62].

The mechanisms by which dynamin affects cortical actin dynamics and actin dynamics during endocytosis are unclear, but some clues have emerged. Dynamin interacts directly with cortactin and mAbp1, two activators of actin filament formation by the Arp2/3 complex as discussed above. Dynamin may also influence the association of N-WASp, an activator of the Arp2/3 complex, or endocytic structures via interactions with syndapins, or intersectin. Dynamin also binds profilins [63], which are multifunctional proteins that bind actin monomers and promote ATP exchange on monomeric actin and filament elongation at barbed ends [64]. Profilin II binds dynamin's PRD and competes with other dynamin-binding partners such as Grb2, the endocytic proteins, amphiphysin and endophilin (W Witke, personal communication). Thus, profilin II could modulate interactions of dynamin with other endocytic proteins that cooperate in coated-vesicle formation. In this way, profilin II may negatively regulate dynamin function. In support of this idea, cells from profilin II-null mice exhibit enhanced membrane turnover (W Witke, personal communication). The interactions of profilin with dynamin and with actin, as well as the participation of dynamin in endocytosis, are all regulated by PIP₂ pointing to a central role for phosphoinositides in

coordinately regulating both endocytic functions and cytoskeletal functions.

Conclusions

The evidence that actin filaments play a role in the function of the endocytic machinery in mammalian cells continues to increase. The major challenges for the future will be to determine the roles played by actin filaments at different steps in the internalization of proteins and fluid, and to determine how the interface of the endocytic machinery and the actin cytoskeleton is structured and regulated. Several candidate proteins that might maintain this interface are now known. Finally, the participation of dynamin in organizing actin filaments at sites other than conventional endocytic zones may be a clue to understanding how cell motility is linked to membrane dynamics.

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