Mini-Review

Molecular Links between Endocytosis and the Actin Cytoskeleton

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The endocytosis process is of critical importance for a variety of cellular life functions. Vesicle formation during receptor-mediated endocytosis involves a complex protein machinery and additional proteins to control it. Although our understanding of this endocytic machinery has grown rapidly during the last decade, little is known about how it interconnects functionally with the cortical cytoskeleton underlying the plasma membrane.

In lower eukaryotes such as yeast, genetic analysis connects the actin cytoskeleton to endocytosis. In mammalian cells, the link is not so convincing. Introducing actin-perturbing drugs (reviewed in Geli and Riezman, 1998) or mutant forms of the Rho family of small GTPases (reviewed in Ellis and Mellor, 2000) disrupts endocytosis in some cells types with some assays, but not in all cases (Gottlieb et al., 1993; Jackman et al., 1994; Lamaze et al., 1997; Fujimoto et al., 2000). Clarification of the role of actin in endocytosis would result if it were possible to identify the molecular players involved and to discover how the proteins of the endocytic machinery are linked functionally to those of the actin cytoskeleton. In this review, we outline the potential roles of the actin cytoskeleton in the endocytic process and examine the experimental evidence for each.

Possible Roles for the Actin Cytoskeleton in Endocytosis

The endocytic process can be broken down into steps, membrane invagination, coated pit formation, coated pit sequestration, detachment of the newly formed vesicle, and movement of this new endocytic compartment away from the plasma membrane into the cytosol. Each step could involve the actin cytoskeleton (Fig. 1, a–e).

Cytoskeletal components may localize the endocytic machinery to domains of the plasma membrane by providing a physical barrier to its diffusion (trapping) or by direct association with components of the machinery (anchoring; Fig. 1 a). The lateral mobility of green fluorescent protein (GFP)[1]-labeled clathrin-coated pits is increased upon

treatment with the actin monomer sequestering drug latrunculin B (Gaidarov et al., 1999) suggesting the removal of a diffusion barrier. In the same study, coated pits formed many times at the same site (Gaidarov et al., 1999). The concept that coated pit formation is initiated at specific and restricted sites was also implied by studies of synaptic vesicle recycling at the *Drosophila melanogaster* neuromuscular junction (Fig. 1 f; Roos and Kelly 1999). Perhaps the sites of endocytosis are linked to the actin cytoskeleton via multifunctional scaffolding proteins.

The actin cytoskeleton could also deform or invaginate the plasma membrane, imposing a curvature on the planar membrane, which makes it easier for coating machinery to pinch off the membrane (Fig. 1 b). Such invaginations, open to small extracellular molecules, but not large ones, were found to be the source of a class of small endocytic vesicles (SLMVs or synaptic-like microvesicles) seen in PC12 cells (Schmidt et al., 1997), but actin has not so far been implicated in such invaginations.

A third possibility is that the cortical cytoskeleton (Fig. 1 g) needs to be removed to allow endocytosis. The rigid cortical actin cytoskeleton has an inhibitory effect on membrane traffic (Trifaró and Vitale, 1993) that could be overcome by an increase in local actin turnover (Fig. 1 c). The immediate vicinity of clathrin-coated pits has been shown to be almost devoid of actin fibers (Fujimoto et al., 2000). However, actin cannot be playing an exclusively negative role since, at the apical surface of polarized epithelial cells, endocytosis was inhibited upon actin depolymerization (Gottlieb et al., 1993), whereas stabilizing actin filaments with jasplakinolide had no effect. Jasplakinolide neither inhibited nor stimulated endocytosis at the apical surface, but stimulated basolateral uptake (Shurety et al., 1998).
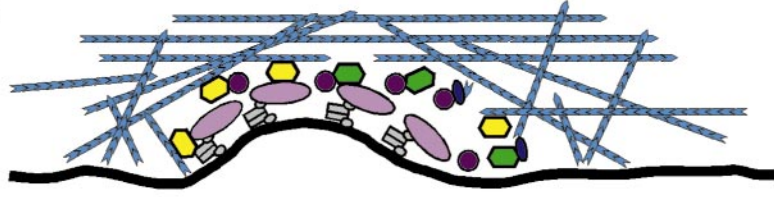
A fourth potential function of the actin cytoskeleton involves the membrane fission event that liberates vesicles from the plasma membrane. When actin polymerization is inhibited in A431 cells by the drug latrunculin A, receptor-mediated endocytosis is arrested at the stage of invaginated coated pits (Lamaze et al., 1997). Thus, it is possible that actin polymerization at the neck provides the force to drive membrane fission or vesicle detachment (Fig. 1 d). Recent actin-depletion studies (Fujimoto et al., 2000) argue against a role of the cortical actin cytoskeleton in the sealing of invaginated vesicles; detachment of clathrin-coated vesicles from the plasma membrane, however, was not measurable in the assay used.

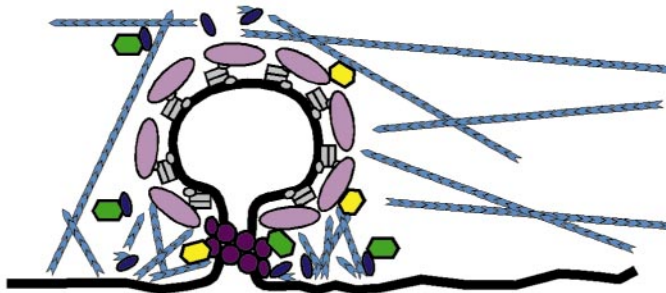Actin may also help drive detached endocytic vesicles

a
Spatial organization
of the endocytic
machinery

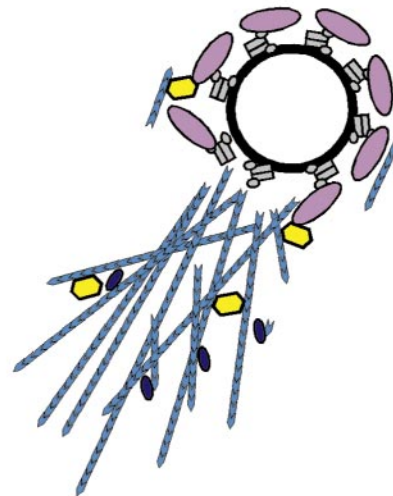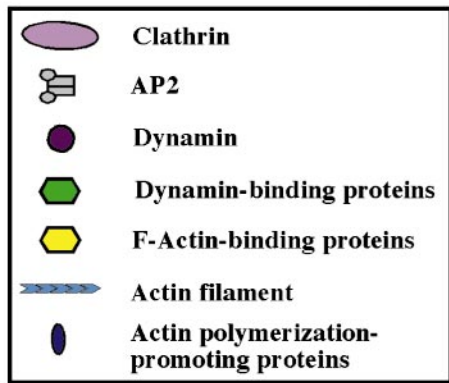**b**
Deformation
and invagination of
plasma membrane

c
Dissolution of the
cortical actin barrier

d
Force generation
during or after the
fission reaction

e
Vesicle movement
into the cytoplasm

f

g

h

Legend:
- Clathrin
- AP2
- Dynamin
- Dynamin-binding proteins
- F-Actin-binding proteins
- Actin filament
- Actin polymerization-promoting proteins

a'
Spatial organization,
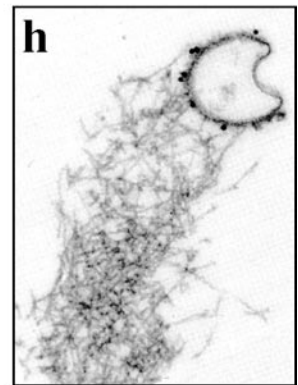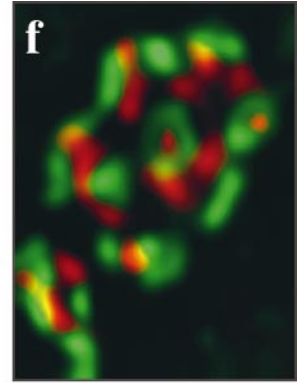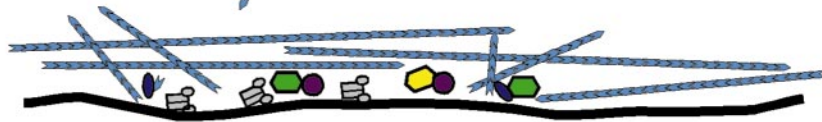i.e. marking of endocytosis
hot spots

Figure 1. Potential roles for the actin cytoskeleton in endocytosis. a–e, The model depicts how the cortical actin cytoskeleton might be involved in different steps of the endocytic process implicating potential functional roles for molecules at the interface of endocytosis and cytoskeletal organization. a, Cytoskeletal structures may organize or constrain the lateral mobility of the machinery for endocytosis. b, Deformation and invagination of the plasma membrane may be supported by the cytoskeleton. c, The cortical actin barrier underlying the plasma membrane might need to be dissolved. d, Actin polymerization may provide force to drive membrane fission during endocytic vesicle formation. e, Actin polymerization may promote the movement of newly formed endocytic vesicles into the cytoplasm by forming a comet tail. The remaining budding site may again be marked by proteins attached to cortical F-actin (a′) and endocytosis can start anew. f–h, Experimental data illustrating some of these hypothetical roles of actin in endocytosis. f, Highly organized endocytic hotspots marked by Dap160 (red) surrounded by sites of exocytosis (green, GluRB) at the *Drosophila* neuromuscular junction (image kindly provided by J. Roos). g, The cortical actin cytoskeleton and three different stages of clathrin-coated pit formation observed by quick freeze deep-etch EM (image kindly provided by J.E. Heuser). h, Electron micrograph of HeLa vesicle in *Xenopus* extracts exhibiting an actin tail and N-WASP immunolabeling at the vesicle surface (Taunton et al., 2000).

through a viscous cytoplasm (Fig. 1 e). In support of this concept, endosomes, pinosomes, clathrin-coated, and secretory vesicles have recently been described associated with actin comet tails in the cytoplasm (Frischknecht et al., 1999; Merrifield et al., 1999; Rozelle et al., 2000), as have endosomes and lysosomes in in vitro systems (Taunton et al., 2000). These comet tails (Fig. 1 h) resemble a mechanism that propels pathogens such as *Listeria monocytogenes* through the cytoplasm of infected host cells. Later stages of endocytosis could also involve actin-based motor proteins (reviewed in Wu et al., 2000). Assuming an F-actin orientation similar to lamellipodia and filopodia, with the fast-growing barbed ends directed towards the plasma membrane, pointed end-directed motor proteins would be required. Recently, the unconventional myosin VI was reported to move towards the pointed end of actin filaments (Wells et al., 1999).

The ways in which the actin cytoskeleton may participate in endocytosis (Fig. 1, a–e) are not mutually exclusive, nor need they be found in all cell types and in all forms of plasma membrane vesiculation.

Mammalian Proteins at the Functional Interface of Actin Organization and Endocytosis

Dynamin and Its Isoforms

Dynamins have been a focus of the endocytosis research since it was demonstrated that the block of endocytosis in the *shibire* mutant of *Drosophila* is due to a mutation in dynamin. This large GTPase controls the fission reaction (reviewed in Sever et al., 2000). Some observations link dynamin to the actin cytoskeleton. When the dynamin K44A mutant, which is defective in GTP binding and blocks endocytosis, is overexpressed the distribution of actin stress fibers and cell shape are altered. Since these changes can also be observed during an endocytosis block induced by K^+-depletion or acidification they may be secondary to the endocytosis defect (Damke et al., 1994; Altankov and Grinnell, 1993). Treatment of primary hippocampal cultures with antisense oligonucleotides to dynamin 1 inhibited neurite outgrowth, an actin-associated process, although again this could be secondary to an endocytosis block (Torre et al., 1994). Dynamin 2aa-GFP was shown to colocalize with filamentous actin at membrane ruffles (Cao et al., 1998). Dynamin 2aa immunoreactivity was also detected at podosomes, cell adhesion structures consisting of actin arrays which often surround a narrow tubular membrane invagination. Overexpression of a GFP-dynamin 2aa mutant similar to the *shibire* mutant of *Drosophila* abolished podosomes (Ochoa et al., 2000). Since another construct, GFP-dynamin2aa K44A, can block endocytosis, but not podosome formation (Ochoa et al., 2000), it can be concluded that the actin rearrangements that occur are not due to secondary effects of an endocytosis block, but that a direct functional link of dynamin to the actin cytoskeleton exists.

Dynamin-interacting Proteins

Dynamin-interacting proteins recognize the COOH-terminal proline-rich domain of dynamin most often via their SH3 domains. Since binding of their SH3 domains can lead to a strong activation of the GTPase activity of dynamin in vitro, dynamin-interacting proteins can additionally be viewed as putative regulators of dynamin.

One class of dynamin-interacting proteins with SH3 domains consists of amphiphysins I and II (reviewed in Wigge and McMahon, 1998). Amphiphysin interacts with AP2 and clathrin, as well as with dynamin, and was proposed to be involved in targeting dynamin to the plasma membrane. Overexpressing the SH3 domains of amphiphysin I and II reduced receptor-mediated endocytosis (Wigge et al., 1997; Owen et al., 1998). Amphiphysin I and II SH3 domains are also known to prevent dynamin ring formation (Owen et al., 1998). Treatment of primary hippocampal cultures with antisense oligonucleotides to amphiphysin I did not affect receptor-mediated and fluid-phase endocytosis, but did inhibit neurite outgrowth and induced a collapse of growth cones (Mundigl et al., 1998). In this case, growth inhibition is unlikely to be secondary to endocytosis perturbations, because no changes in endocytosis were detected. Mundigl et al. (1998) were not able to show a direct interaction of amphiphysin I with G- or F-actin; therefore, this effect may involve currently unknown actin-regulating proteins.

A second family of dynamin-interacting proteins, which has been found to exhibit functional links to the actin cytoskeleton, are the syndapins (Qualmann et al., 1999; Qualmann and Kelly, 2000). Genes of mouse orthologs of syndapins were also described and termed PACSINs (Ritter et al., 1999). Whereas syndapin I is a brain-specific protein, syndapin II isoforms show a broader tissue distribution. Both forms of this SH3 domain-containing protein interact with dynamins, synaptojanins, and synapsins, all proteins that are involved in membrane trafficking. Additionally, syndapins interact with N-WASP, a more ubiquitously expressed, brain-enriched isoform of the Wiskott-Aldrich syndrome protein, a potent activator of the Arp2/3 complex actin polymerization machinery (Qualmann et al., 1999; Qualmann and Kelly, 2000). In line with their protein associations, syndapins affect both endocytosis and the actin cytoskeleton. The SH3 domain of both syndapin isoforms inhibited receptor-mediated internalization of transferrin in vivo (Qualmann and Kelly, 2000) at a step that occurs after constricted pit formation, as demonstrated in an in vitro reconstitution system (Simpson et al., 1999). These results are consistent with the fact that syndapins interact with dynamin. Overexpression of full-length syndapins had a strong effect on cortical actin organization, inducing filopodia. This phenotype appears to be mediated by the Arp2/3 complex at the cell periphery because it was completely suppressed by coexpression of a cytosolic COOH-terminal fragment of N-WASP (Qualmann and Kelly, 2000). Whereas overexpressing the SH3 domain alone was sufficient to block endocytosis, the full-length protein was necessary to induce filopodia, suggesting that syndapin-induced cytoskeletal rearrangements are not an indirect consequence of the inhibition of receptor-mediated endocytosis. Syndapins also localized to sites of high actin turnover, such as filopodia tips and lamellipodia, and a splice variant of a chicken ortholog of syndapin, FAP52, was described as focal adhesion protein (Meriläinen et al., 1997). Syndapins could thus couple actin po-

lymerization to dynamin's function in the fission reaction. They may be able to trigger a burst of actin polymerization around the vesicle neck that is required to detach the newly formed vesicle from the membrane or to move it into the cytosol (Fig. 1, d and e). In similar tails formed by the pathogen *Listeria moncytogenes*, actin filaments are oriented with their barbed, fast growing ends towards the vesicle and WASP proteins are also localized to this interface. To fulfill such a role, syndapins, however, would have to use the same SH3 domains to interact with both dynamin and N-WASP. Since syndapins have oligomerization domains and other protein interaction interfaces, simultaneous interactions may well be possible or additional proteins may be involved. Alternatively, these interactions may be more dynamic and dynamin rings may increase the local concentration of the syndapins.

Proteins that Interact with the Endocytic Machinery and the Actin Cytoskeleton

In many cases, both endocytosis and actin filament organization in yeast are sensitive to perturbations in the same gene products. Recently, mammalian homologues of two such bifunctional yeast proteins, Abp1 and Sla2p, have been identified. The mammalian Sla2 homologue, HIP1R (Huntingtin interacting protein 1-related; Seki et al., 1998), has been shown to be stably associated with clathrin-coated pits and vesicles (Engqvist-Goldstein et al., 1999). The subcellular distribution of HIP1R overlaps with clathrin both at the cell cortex and in the perinuclear region. GFP-HIP1R fusion proteins in living cells behave like clathrin-GFP, associating with newly formed coated pits and vesicles that bud from the plasma membrane (Engqvist-Goldstein et al., 1999; Gaidarov et al., 1999). The COOH-terminal talin-like domain of HIP1R binds F-actin in vitro and is important for the colocalization of the protein with cortical actin in vivo, whereas the association with clathrin-coated pits is mediated by the NH$_2$-terminal half of the protein (Engqvist-Goldstein et al., 1999). HIP1R might therefore represent a physical link between F-actin and clathrin-coated endocytic structures, a function that may be required for spatial organization of endocytosis (Fig. 1 a), for an actin-dependent movement of newly formed coated vesicles (Fig. 1 e), or both.

Thus far, mainly the cytoskeletal interactions of the mouse homologue of yeast Abp1, mAbp1, have been characterized. mAbp1 specifically binds to F-actin in vitro and in vivo using two different functional domains and is preferentially associated with dynamic actin structures (Kessels et al., 2000). Its involvement in actin dynamics is also suggested by the fact that mAbp1, primarily a cytosolic protein, accumulates in lamellipodial areas of cell growth and in lamellipodial sheets of spreading and moving cells (Kessels et al., 2000). This function is dependent on the GTPase Rac1. mAbp1, initially named SH3P7 (Sparks et al., 1996), was also identified as a Src kinase substrate (Lock et al., 1998; Larbolette et al., 1999). We have recently discovered that the mAbp1 SH3 domain interacts with dynamin in vitro and in vivo, and that overexpression of the COOH-terminal SH3 domain of mAbp1 leads to a drastic reduction of the receptor-mediated endocytosis in vivo (Kessels, M.M., Å.E.Y. Engqvist-Goldstein, D.G.

Drubin, and B. Qualmann, unpublished results). Thus, mAbp1 can bind to the endocytic machinery via its SH3 domain and to the actin cytoskeleton via its two NH$_2$-terminal actin-binding modules and may therefore physically link the endocytic machinery to the cortical F-actin (Fig. 1 a). It could also participate in the proposed initiation of actin tail formation, coordinating it temporally and spatially with dynamin function (Fig. 1 d), or may also function within the putative actin tails of endocytic vesicles (Fig. 1 e).

The members of the family of WASPs are all multidomain proteins essential for the regulation of actin dynamics in a variety of species. Wiskott-Aldrich syndrome patients show a decrease in the number of microvilli and abnormal pattern of actin filaments in platelets and lymphocytes (reviewed in Snapper and Rosen, 1999). WASPs greatly enhance the ability of the Arp2/3 complex to nucleate actin filaments (reviewed in Welch, 1999), binding Arp2/3 via the extreme COOH terminus of WASP (Machesky and Insall, 1998). The ubiquitous, but highly brain-enriched relative of WASP, N-WASP, is activated by phosphatidylinositol (4,5) bisphosphate and Cdc42 binding (Rohatgi et al., 1999), and induces extremely long actin microspikes when coexpressed with active Cdc42 (Miki et al., 1998). Mammalian WASPs and the yeast homologue Las17 are additionally implicated in endocytosis. EGF treatment of COS7 cells induced N-WASP to form a complex with the EGF receptor, most likely via the adaptor protein Grb2 (Miki et al., 1996). Lymphocytes from WASP knockout mice exhibited both a reduction in actin polymerization and defects in T cell receptor endocytosis (Zhang et al., 1999). Furthermore, in brain extracts, N-WASP is associated with syndapin I (Qualmann et al., 1999), which is likely to play a role in receptor-mediated endocytosis (Simpson et al., 1999; Qualmann and Kelly, 2000). A specific recruitment of N-WASP to the dynamin neck of a constricted pit could allow an N-WASP–promoted local F-actin assembly, first supporting the pinching-off process and subsequently leading to propulsion of the vesicle through the cytoplasm (Fig. 1, d and e). In support of this hypothesis, Taunton et al. (2000) reported that endosomal and lysosomal vesicles moved via actin propulsion in cell-free *Xenopus laevis* extracts. These vesicles exhibited N-WASP staining at the interface of the actin tail and the vesicle (Fig. 1 h).

Actin is a slow ATPase, and the nucleotide exchange reaction is an important point of regulation. The ADP/ATP exchange of actin is catalyzed by the small G-actin binding protein profilin, which is an important promoter of actin dynamics in vivo (reviewed in Schluter et al., 1997). Profilins also bind to the Arp2/3 complex and to polyproline stretches of the WASP and family proteins ena/VASP. The brain-enriched profilin II isoform was recently shown to be able to interact with dynamin and synapsins by affinity chromatography, suggesting a role for profilin II in membrane trafficking events in the brain (Witke et al., 1998). In a perforated PC12 cell system, the addition of recombinant profilins I and II to diluted cytosol stimulated the biogenesis of synaptic-like microvesicles (Schmidt and Huttner, 1998*)*. The ability of profilins to enhance nucleotide exchange on actin can be regulated by phosphoinositides. The binding of phosphoinositides to profilin causes

actin to dissociate from profilin. Witke et al. (1998) suggested a model in which profilin recruits both actin and potential ligands to phosphoinositide-rich membrane areas and thus facilitates the spatially coordinated accumulation of signaling molecules, endocytosis machinery proteins, and nascent actin polymerization (Fig. 1, a, d, and e).

Phosphoinositides directly affect signaling processes, membrane trafficking events, and the actin cytoskeleton (reviewed in De Camilli et al., 1996; Martin, 1998). The polyphosphoinositide phosphatase synaptojanin 1 (McPherson et al., 1996), which catabolizes PIP_2 and PIP_3 (Woscholski et al., 1997), and interacts with several SH3 domain-containing accessory proteins implicated in clathrin-mediated endocytosis, may therefore be another attractive candidate to act at the interface of endocytosis and actin dynamics. Synaptojanin 1 plays a role in the uncoating of endocytic coated vesicles. In neurons of synaptojanin 1-deficient mice, clathrin-coated vesicles accumulate in nerve endings and synaptic depression during prolonged high-frequency stimulation is enhanced (Cremona et al., 1999). Considering the pleiotropic roles of phosphoinositides, the cellular function of synaptojanin might not be limited to the regulation of membrane traffic. A decrease in the amount of PIP_2, a potent regulator of actin-binding proteins, is believed to lead to local actin depolymerization (see Sakisaka et al., 1997). Synaptojanin has been shown to be able to hydrolyze PIP_2 that is bound to actin-binding proteins such as profilin, cofilin, and α-actinin in vitro. In COS-7 cells, overexpression of synaptojanin, but not a phosphatase-negative mutant, caused rearrangement of stress fibers (Sakisaka et al., 1997). Thus, synaptojanin might promote local actin turnover (Fig. 1 c) and uncoating in parallel, or vesicle uncoating may be coordinated temporally with the depolymerization of vesicle-associated actin.

Perspectives

In this review we propose that several proteins that interact with components of the endocytic coat or with dynamin, even the GTPase dynamin itself, directly or indirectly affect actin cytoskeletal organization. An important remaining question is whether these proteins mediate both endocytosis and the actin organization in parallel, or whether their function in endocytosis requires their interaction with the cortical actin cytoskeleton. The actin cytoskeleton has been implicated in endocytosis for many years, but we have had few clues to the underlying mechanism. Several hypotheses are plausible (Fig. 1), but we do not know which one, or even if more than one, will be validated by further experiments. Indeed, the complexity of the results obtained with actin stabilizing and destabilizing drugs may be explained if actin has both inhibitory and facilitatory actions.

The situation is rapidly clarifying because of the availability of dominant negative fragments of endocytic machinery and actin cytoskeleton proteins. At the same time, in vitro assays of budding and observations of budding in real time of GFP-labeled vesicles offer unprecedented opportunities to dissect out the steps of endocytosis. Actin and endocytosis studies are clearly engaged to each other; the final marriage can be expected soon.

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