The Actin Cytoskeleton Is Required for Receptor-mediated Endocytosis in Mammalian Cells*

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Actin filament organization is essential for endocytosis in yeast. In contrast, the actin-depolymerizing agent cytochalasin D has yielded ambiguous results as to a role for actin in receptor-mediated endocytosis in mammalian cells. We have therefore re-examined this issue using highly specific reagents known to sequester actin monomers. Two of these reagents, thymosin β4 and DNase I, potently inhibited the sequestration of transferrin receptors into coated pits as measured in a cell-free system using perforated A431 cells. At low concentrations, thymosin β4 but not DNase I was stimulatory. Importantly, the effects of both reagents were specifically neutralized by the addition of actin monomers. A role for the actin cytoskeleton was also detected in intact cells where latrunculin A, a drug that sequesters actin monomers, inhibited receptor-mediated endocytosis. Biochemical and morphological analyses suggest that these reagents inhibit later events in coated vesicle budding. These results provide new evidence that the actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells.

The plasma membrane is directly linked to and functionally integrated with the underlying actin-based cytoskeleton which forms the cell “cortex.” Thus, it might be anticipated that vesicular trafficking at the plasma membrane would require the active rearrangement of cortical actin filaments to remove a barrier to vesicular fusion or budding events. Alternatively, actin and actin-based motor proteins might be required to direct vesicle budding and fusion events through the cell cortex at the plasma membrane. Actin filaments can play both inhibitory and facilitatory roles in exocytosis. For example, agonist-stimulated secretion appears to require localized disassembly of F-actin at the cell periphery (1, 2). Furthermore, low concentrations of proteins which sequester actin monomers can trigger regulated secretion in permeabilized pancreatic acinar cells suggesting that actin filaments might act as a clamp preventing fusion of docked vesicles (3). In contrast, higher concentrations of these actin-sequestering proteins inhibit regulated secretion, suggesting that actin filament integrity might also play an as yet undefined, facilitory role in regulated exocytosis (3).

Receptor-mediated endocytosis of the mating pheromone α-factor is potently inhibited in the yeast, S. cerevisiae, expressing mutations in either actin or the actin-binding protein, fimbrin (4). Yeast carrying mutations in three other genes, END3, END5/VRP1, and END7/RVS167, which disrupt actin organization, were also shown to be defective in endocytosis (5, 6). More recently a role for the type I myosin, myo5, in receptor-mediated endocytosis in yeast was revealed (7). Together these studies establish that actin filaments and actin-based motor proteins play an essential role in endocytosis in yeast.

In contrast, the role of actin in endocytosis in mammalian cells remains poorly understood. Cytochalasin D, a drug that destabilizes actin filaments, inhibits receptor-mediated and fluid-phase endocytosis at the apical surface of polarized Madin-Darby canine kidney cells (8) and Caco 2 cells (9), but has no effect on endocytosis at the basolateral surface. There are conflicting results on the effects of cytochalasin D on receptor-mediated endocytosis of transferrin in nonpolarized cells (10–13). Cytochalasin D caps the growing ends of actin filaments and thus causes the depolymerization of actin filaments that are actively turning over, i.e. predominantly the stress fibers. In contrast, cortical actin filaments are more resistant to disruption by cytochalasin D (15), providing a possible explanation for negative results using this reagent. Other lines of evidence have recently implicated actin filament organization in endocytosis. For example, activation of the Rho family GTPases, which trigger actin filament assembly at the cortex, has been shown to stimulate fluid phase pinocytosis (16, 17) but inhibit clathrin-mediated endocytosis (12). These findings prompted us to re-examine the role of actin microfilaments in receptor-mediated endocytosis using reagents which selectively sequester actin monomers, thereby disrupting actin filaments by shifting the equilibrium to the depolymerized state. Here we report that these actin-binding proteins or drugs inhibit the formation of clathrin-coated vesicles at the plasma membrane. These results provide new evidence that the actin cytoskeleton plays an essential role in receptor-mediated endocytosis in mammalian cells.

MATERIALS AND METHODS

Cells and Reagents—A431 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% defined fetal calf serum (HyClone) as described previously (28, 29). Following trypsinization, 4 × 10⁶ cells were seeded onto 15-cm culture dishes 20–24 h prior to preparing perforated cells, also as described previously (28, 29). Biotinylated transferrin (B-Tfn) was prepared (18, 29) using either sulfo-NHS-XS-biotin (6-(6-(biotinoyl)amino)hexanoyl)amino)hexanoic acid, sulfosuccinimidyl dextr, sodium salt) obtained from Molecular Probes (Eugene, OR) or sulfo-NHS-SB-biotin (sulfosuccinimidyl 2-biotinamido)ethyl-1,3-dithiopropionate) from Pierce. The former biotinylating reagent was used, in general, for avidin sequestration assays as the longer spacer arm gave lower background signals. The latter cleavable biotinylating reagent was required for the MesNa (β-mercaptoethanesulfonic acid) assay.

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1 The abbreviations used are: B-Tfn, biotinylated human dipher ferrin; TfnR, transferrin receptors; Tβ4, thymosin β4; MesNa, β-mercaptoethanesulfonic acid.
Thymosin β4 was expressed in *Escherichia coli* and purified to >99% homogeneity (based on Coomassie Blue-stained gels) as described (19). Monomeric actin was prepared from rabbit skeletal muscle (3). Latrunculin B was obtained from Alexis Corp. (San Diego, CA), latrunculin A was from Molecular Probes, DNase I was from Boehringer Mannheim, and cytochalasin D was from Sigma. All other materials were reagent grade.

**Cell-free Assays for Receptor-mediated Endocytosis—**Perforated A431 cells were prepared and assays were performed exactly as described previously (18, 29). For assays containing Tβ4 or DNase I, perforated cells were incubated with the drugs at 4 °C for 10 min before the addition of the cytosol. This treatment appeared to enhance the effects of DNase I and Tβ4. To block the effects of DNase I, actin monomers were added to the perforated cells at 4 °C for 10 min before the addition of DNase I. Tβ4 was inactivated *in vitro* by formation of Tβ4-actin complexes as described (3). Briefly, 7 μl of 160 μM Tβ4 was incubated with 42 μl of 180 μM G-actin (Tβ4:actin molar ratio of 1:5) for >1 h on ice and added to perforated cells.

**Receptor-mediated Endocytosis in Intact Cells—**Adherent A431 cells were dissociated for 5 min at 37 °C in phosphate-buffered saline containing 5 mM EDTA, washed in serum-free culture medium containing 0.2% bovine serum albumin and 20 mM Hepes, pH 7.2 (SFM), and resuspended at 2 × 10^6 cells/ml in SFM at 4 °C. Cells were then diluted 10-fold into SFM containing the indicated concentrations of latrunculin A or B (prepared as a 2 mg/ml stock solution and stored at 4 °C) and incubated at 37 °C for 1 h. Upon return to ice, B-Tfn was added (to 2 μg/ml) from a 20 μM stock. Aliquots (50 μl) were removed and kept on ice to determine total surface bound ligand and 4 °C controls, and the remaining suspension was returned to 37 °C. Aliquots were removed after increasing times to determine the kinetics of endocytosis. B-Tfn internalization was measured using the avidin protocol as described (29). Similar results were obtained if adherent cells were pretreated with latrunculin B before their release from the plate by phosphate-buffered saline/EDTA for endocytosis assays in suspension. Control experiments established that at the concentrations used, the solvent alone had no effect on endocytosis.

**Electron Microscopy—**Anti-human Tfn-R antibody HTR-D65 (obtained from I. Trowbridge, Salk Institute, La Jolla, CA) was conjugated to 10-nm gold particles (BBI International) as described previously (28). Incubations for morphological studies with intact cells were performed exactly as described for biochemical analysis except that they were scaled up 6-fold. After pelleting, cells were resuspended in 0.2 M cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and processed for conventional epon sectioning as described (28). Samples were viewed on a Jeol 1200 at 60 kV. Quantitation of gold particles was performed at the microscope by random examination of cell profiles at a magnification of 20,000.

**RESULTS AND DISCUSSION**

**Biphasic Effects of Thymosin β4 on Receptor-mediated Endocytosis—**Thymosins are abundant and highly specific actin monomer-binding proteins ubiquitously expressed in vertebrate cells (21). Tβ4 forms a 1:1 complex with α-actin and sequesters actin monomers (22). As a result, actin monomers are not available for polymerization, and actin filaments are depolymerized. Although Tβ4 can directly interact with and depolymerize F-actin at high concentrations, at lower concentrations (<20 μM) it can neither sever nor cap actin filaments (23). Recent studies on the effects of Tβ4 on secretion in permeabilized acinar cells established the specificity of this reagent for disruption of actin filaments (3). Since Tβ4 disrupts actin filaments by a mechanism completely distinct from that of cytochalasin D, we tested its effects on receptor-mediated endocytosis in perforated A431 cells. Receptor-mediated endocytosis in this cell-free assay is dependent on cytosol and an ATP-regenerating system and is detected by the sequestration (either in constricted coated pits or sealed coated vesicles) of receptor-bound biotinylated ligands from exogenously added avidin (24, 29).

Titration of Tβ4 into perforated A431 cells showed that it had a biphasic effect on receptor-mediated endocytosis. Tβ4 concentrations up to 0.5 μM stimulated ligand sequestration by ~50% (Fig. 1, A and inset), whereas higher concentrations led to a complete inhibition of endocytosis (half-maximum inhibi-

**FIG. 1.** The actin-monomer-binding proteins thymosin β4 and DNase I inhibit receptor-mediated endocytosis in perforated cells. The sequestration of B-Tfn into constricted coated pits or sealed coated vesicles was measured in perforated A431 cells by its inaccessibility to exogenously added avidin as described under “Materials and Methods.” Panel A, assays were performed in the presence of the indicated concentrations of thymosin β4. The inset shows an expanded view of the stimulatory effects seen at low concentrations of Tβ4. ATP- and cytosol-dependent sequestration is expressed as a percentage of total cell-associated ligand. The data represent averages (± S.D.) of 9 experiments. Panel B, Tβ4, at the indicated concentrations, was preincubated with or without 5-fold molar excess of monomeric actin for 1 h at 4 °C prior to addition to perforated cells. Preincubation with actin abrogated both the stimulatory and inhibitory effects of Tβ4; actin on its own had no effect. The data represent averages (± S.D.) of 5 experiments. Panel C, sequestration assays were performed in the presence of the indicated concentrations of DNase I in the absence (●) or presence (○) 10 μM monomeric actin. The data represent averages (± S.D.) of three experiments.

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hition seen at higher concentrations were abrogated (Fig. 1B). Exogenous actin on its own had no effect on receptor-mediated endocytosis. Together these results suggested involvement of the actin cytoskeleton in receptor-mediated endocytosis.

**Effect of DNase I on Receptor-mediated Endocytosis**—DNase I is a structurally distinct actin-sequestering protein with an exceptionally high affinity ($K_d \sim 1$ nM) for monomeric actin. DNase I is characterized by its unique ability to increase the depolymerization rate constant of actin at the pointed filament end without severing the actin filaments (25). As a result, the depolymerization of filaments capped at their barbed ends is accelerated, and actin monomers are sequestered. We therefore examined the effects of DNase I on receptor-mediated endocytosis in perforated cells and found that it also potently inhibited the sequestration of transferrin into coated pits (Fig. 1C, open circles). Inhibition by DNase I was concentration-dependent with half-maximal inhibition occurring at $<5 \mu M$. In contrast to Tβ4, no stimulation of endocytosis could be observed at low concentrations of DNase I. However, this could be explained by the difference in affinity for actin seen between DNase I and Tβ4 (1 nM versus $1 \mu M$, respectively) and the differences in their effects on actin filament depolymerization. Again, the specificity of DNase I action on the actin network was confirmed since its inhibitory effects on endocytosis were neutralized in the presence of actin monomers (Fig. 1C, closed circles). Together with the effects seen with Tβ4, these results suggest that actin filaments are required for receptor-mediated endocytosis in perforated mammalian cells.

**Effect of Latrunculins on Intact A431 Cells**—The perforated A431 cell system has been extensively characterized biochemically and morphologically and appears to faithfully reconstitute many of the biochemically distinct events required for the formation of endocytic clathrin-coated vesicles (12, 18, 20, 24, 28). Nonetheless, it remained possible that the requirement for actin assembly seen in perforated cells might reflect an artificial effect due to disorganization of the actin cytoskeleton as a result of the mechanical disruption of the plasma membrane. Therefore, we examined the effect of latrunculin A on receptor-mediated endocytosis in intact A431 cells. Latrunculins are a new class of membrane-permeable, actin-disrupting agents which show powerful and specific effects on the actin-based cytoskeleton of nonmuscle cells (26). In *vitro*, latrunculin affects the kinetics of polymerization of actin by forming a nonpolymerizable 1:1 molar complex with G-actin (27). Thus, like Tβ4 and DNase I but unlike the cytochalasins, latrunculins destabilize actin filaments by sequestering actin monomers and shifting the equilibrium to the disassembled state.

To determine whether actin filaments are required for receptor-mediated endocytosis in intact cells, A431 cells were exposed to increasing concentrations of latrunculin A for 1 h. This treatment induced dramatic changes in the morphology of adherent A431 cells which became round and contracted. As described previously (28) these morphological effects were similar to those seen when cells were incubated with 10 μg/ml cytochalasin D (data not shown). The morphology of A431 cells in suspension was not dramatically altered by latrunculin A treatment. The data in Fig. 2A show that latrunculin A significantly inhibited the rate of receptor-mediated endocytosis in intact cells in a concentration-dependent manner. The extent of inhibition (~50%) was consistent with those reported for the effects of cytochalasin D treatment in Hep2 cells (14). Half-maximal inhibition was obtained at ~4 μg/ml latrunculin A. While these concentrations are somewhat higher than those needed to destabilize stress fibers, they are not inconsistent with destabilization of more resistant elements of the cortical actin network. Latrunculin B had similar effects although at slightly higher concentrations (not shown; but see below), consistent with this analogue being less potent. As previously shown (11, 12), cytochalasin D (10–50 μg/ml) had no effect on endocytosis in A431 cells.

TfnR undergo constitutive endocytosis and recycling. As a result, the number of surface TfnR reflects the relative rates of internalization and recycling. As can be seen in Fig. 2B, cells incubated with latrunculin A show a concentration-dependent increase in surface TfnR. Thus, the effects of latrunculin A on intact cells are consistent with an inhibition in endocytosis without a concomitant effect on TfnR recycling. A similar finding was reported following cytochalasin D treatment in Hep2 cells (14). Together, these results confirm a requirement for actin in receptor-mediated endocytosis both in intact and perforated mammalian cells.

**Actin Filaments Are Not Required for Clustering of TfnR into Coated Pits**—Efficient receptor-mediated endocytosis requires both the concentration of receptor-bound ligands into coated pits and the subsequent budding of coated vesicles. Thus, it remained possible that coated vesicle formation continued in the presence of these actin-monomer sequestering agents, but TfnR clustering in coated pits was impaired. To test this we used the gold-conjugated anti-Tfn monoclonal antibody D65 and conventional thin section electron microscopy to examine the distribution of TfnR relative to coated pits in A431 cells treated with or without 25 μM latrunculin B. This treatment resulted in a 40% inhibition of TfnR endocytosis measured in parallel using biochemical assays. The micrographs in Fig. 3 show that neither the morphology of coated pits nor their ability to cluster TfnR was affected. Quantitation of these results showed that 37% of surface D65-gold was found associated with coated pits in both control (100 of 270 gold particles counted) and latrunculin B-treated (114 of 308 gold particles counted) samples.
Actin Filaments Are Required for Clathrin-coated Vesicle Budding—Confirmation that actin filaments were required for coated vesicle formation was obtained using the perforated cell assay system to selectively measure the budding of preformed coated pits. Previous characterization of this system has established that detachment of preformed coated pits can be measured selectively using the small membrane-impermeant reducing agent MesNa as a probe for the internalization of biotinylated ligands into sealed coated vesicles (24, 28). Using this assay the formation of constricted coated pits and coated vesicle budding were detected when receptor-bound biotinylated ligands were internalized into sealed vesicles that are inaccessible to MesNa. Using the MesNa assay, we again found a biphasic response to thymosin β4. The data in Fig. 4 show that ligand internalization, like sequestration, was stimulated at low concentrations and inhibited at higher concentrations of Tβ4. The inhibition seen at high concentrations of Tβ4 is consistent with the results of ultrastructural analysis and suggest that actin filaments are required for late events (either for coated pit constriction, coated vesicle detachment or both) in endocytic coated vesicle formation. The stimulation of coated vesicle budding seen at low concentrations of Tβ4 could reflect destabilization of actin filaments that otherwise act as a barrier to vesicle budding and detachment as observed for exocytosis (3).

In summary, the use of highly specific actin modulatory proteins that sequester actin monomers has revealed a requirement for actin filaments in receptor-mediated endocytosis in mammalian cells. These results resolve apparent discrepancies and suggest a similarity in the mechanisms of receptor-mediated endocytosis in yeast and mammalian cells. It will be important to determine whether the actin requirement in mammalian cells reflects a direct involvement of actin filaments in coated vesicle budding or instead reflects a more general requirement for the structural integrity of the cell cortex in plasma membrane function. Additional evidence for the involvement of other actin binding proteins or actin-based type I myosins, as appears to be the case for endocytosis in yeast (4, 7), may help to distinguish these two possibilities.

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![Fig. 3. TfnR clustering into coated pits is unaffected by latrunculin B.](image-url)
