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Actin-, myosin- and ubiquitin-dependent endocytosis

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Abstract. Endocytosis is a general term that is used to describe the internalization of external and plasma membrane molecules into the cell interior. In fact, several different mechanisms exist for the internalization step of this process. In this review we emphasize the work on the actin-dependent pathways, in particular in the yeast *Saccharomyces cerevisiae*, because several components of the molecular machinery are identified. In this yeast, the analysis of endocytosis in various mutants reveals a requirement for actin, calmodulin, a type I myosin, as well as a number of other proteins that affect actin dynamics. Some of these proteins have homology to proteins in animal cells that are believed to be involved in endocytosis. In addition, the demonstration that ubiquitination of some cell surface molecules is required for their efficient internalization is described. We compare the actin, myosin and ubiquitin requirements for endocytosis with recent results found studying these processes using *Dictyostelium discoideum* and animal cells.

Key words. Ubiquitin; yeast; *Saccharomyces cerevisiae*; *Dictyostelium discoideum*; cytoskeleton; mutants; endocytosis; actin; myosin; calmodulin.

Endocytosis is the pathway whereby cells internalize extracellular molecules and portions of their own plasma membrane and has been shown to occur in all eukaryotes examined thus far. The most studied endocytic systems are in animal cells where several different types of endocytosis exist. The uptake of particles, such as bacteria or protozoal pathogens, has been termed phagocytosis and was first visualized about a century ago by light microscopy. Light microscopy was also used to visualize the uptake of fluid, and this process was termed pinocytosis. Distinct mechanisms of pinocytosis, distinguished by the characteristics of the internalization step, have subsequently been uncovered. The term 'macropinocytosis' has been coined to describe the internalization of fluid into large vacuoles. In many cases this internalization event has been linked to the process of membrane ruffling, where membrane extensions, filipodia, fold back onto and fuse with the plasma membrane, thereby entrapping a certain amount of extracellular fluid. Small aliquots of fluid can also be internalized by a process called micropinocytosis, a term used to describe a variety of processes including adsorptive internalization events. The most specific and best characterized of the latter events is receptor-mediated endocytosis. In this pathway, ligands bound to receptors are internalized at specific sites on the plasma membrane through invaginations that bud from the membrane to form vesicles. When these vesicles form, small amounts of fluid are nonspecifically enclosed and internalized.

The different types of endocytosis are mediated by different internalization mechanisms. The uptake of particles by phagocytosis requires actin polymerization, calcium, and is influenced by phosphorylation events [1, 2]. The process of macropinocytosis also requires actin, which is important to control the shape and dynamics of membrane extensions. The actin dynamics are regulated by small guanosine triphosphatases (GTPases) of the rac and rho family [3]. Several mechanisms for receptor-mediated endocytosis probably exist. The best characterized pathway of internalization is via clathrin-coated vesicles. In this pathway, the polymerization of clathrin triskelions onto the membrane and/or their dynamic rearrangements are thought to be the driving force to form a membrane invagination. Receptors are included into clathrin-coated pits through the interaction of specific signals in their cytoplasmic tails with clathrin adaptor proteins that lie between the clathrin triskelions and the membrane [4]. Another protein, dynamin, a GTPase found associated with clathrin-coated structures, is implicated in the late stages of vesicle formation after membrane invagination [5–7]. Actin apparently plays no role in clathrin-mediated internalization from the basolateral surface in animal cells, but inhibitors of actin polymerization do affect clathrin-mediated endocytosis from the apical surface [8].

Some toxins have been shown to be internalized by clathrin-independent pathways [9]. In these studies some inhibition of internalization by the actin polymerization inhibitors, the cytochalasins, is observed, consistent with

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a role for actin. The molecular requirements for these pathways are not clear. An interesting alternative route for internalization has been uncovered through the study of the internalization of cross-linked glycosylphosphatidylinositol (GPI)-anchored proteins. GPI-anchored proteins in some cell types are reported to be internalized through non-clathrin-mediated mechanisms and sometimes apparently involve caveolae, small cell surface invaginations that are enriched in a protein called caveolin [10, 11]. Internalization of GPI-anchored proteins is inhibited by cytochalasins [12], and agents that deplete cholesterol from the cells cause a cell surface accumulation of cross-linked GPI-anchored proteins [13], suggesting a role for actin and cholesterol. These processes may also be influenced by phosphorylation events [12, 14].

The studies of endocytosis in other eukaryotes have focused largely on the slime mold, *Dictyostelium discoideum* and the yeast, *Saccharomyces cerevisiae*, where genetic approaches have been possible. Like animal cells, *D. discoideum* has both phagocytic and pinocytic mechanisms. Phagocytosis is required for growth on medium where bacteria are the principal source of nutrients. Pinocytosis is apparently important for growth on axenic medium. Rates of pinocytosis are lower in a clathrin heavy chain mutant [15], suggesting an involvement of clathrin in this organism as well. Mutants defective in multiple type I myosins are also defective for pinocytosis (see below), suggesting that actin may be required for pinocytosis in this organism.

Both fluid-phase and receptor-mediated endocytosis have been studied in *S. cerevisiae* and the remaining part of this review will focus largely on studies of this organism. Fluid-phase endocytosis can be followed using the fluorescent dye, lucifer yellow (LY) [16], whereas receptor-mediated endocytosis can be followed using the yeast pheromone, α-factor. α-Factor binds to the product of the *STE2* gene [17] and is internalized by a time-, temperature- and energy-dependent process [18, 19]. Once internalized, α-factor and the α-factor receptor (Ste2p) pass through at least two morphologically distinct, biochemically separable compartments, called early and late endosomes [20, 20a] on their way to the vacuole, where they are degraded [21, 22]. Several other proteins are believed to follow the same pathway, including the a-factor receptor [23, 24], several permeases [25–27] and adenosine triphosphate binding cassette (ABC) transporters [28–30]. The yeast endocytic pathway and the vacuole biogenesis pathways overlap at least from the prevacuolar/late endosomal compartment to the vacuole [31]. Recently, the sterol dye, FM-4-64, has been shown to insert into the plasma membrane from where it is delivered to the yeast vacuole, probably via the same endocytic pathway [32]. To date, no evidence for multiple endocytic internalization mechanisms in yeast has been published.

Isolation of endocytosis mutants

Analysis of yeast mutants has led to the identification of gene products required for many cellular processes. Initial screens for endocytosis-deficient (*end*) mutants started with a bank of mutants that are temperature-sensitive (Ts-) for growth. *end* mutants were then identified by assaying individual isolates for accumulation of LY in the vacuole or internalization of radiolabelled α-factor [18, 33]. The latter screen resulted in the isolation of the first yeast mutants specifically affected in receptor-mediated endocytosis, *end3-1* and *end4-1* [33]. Both mutants are also defective in accumulation of LY in the vacuole, indicating that fluid-phase endocytosis is also affected in the mutants. Outcrosses of these mutants showed that in each case End$^-$ and Ts$^-$ cosegregated, indicating that both phenotypes are caused by the same mutation. This established the validity of such a screening approach. *end* mutants represented about 1–2% of the total mutants in the Ts$^-$ bank.

Further screening with this approach yielded four more Ts$^-$ *end* mutants in which both receptor-mediated and fluid-phase endocytosis are blocked [34]. One of the new mutants is a second allele of *END4* (*end4-2*), while the *end5-1*, *end6-1* and *end7-1* mutants are affected in new *END* genes. The *end6-1* mutation maps to the previously reported *r*educed *v*iability upon *s*tarvation 161 (*RVS161*) gene [35] and is caused by an arginine 59 to lysine change (A.M., unpublished results). Cloning by complementation identified the genes affected in the *end5-1* and *end7-1* mutants as *VRP1* and *ACT1*, respectively. The mutation found in *end7-1* is an aspartic acid replacement of glycine [34] which affects the DNAse I binding loop of actin.

The *RVS161* gene product is also required for correct actin cytoskeleton localization, and disruption of *RVS161* leads to a defect in α-factor internalization similar to that in *end6-1* mutant cells [34]. The *RVS161* gene product shows significant amino acid homology to the mammalian synapse-specific protein amphiphysin, which may play a role in synaptic membrane recycling [36] and to a yeast gene, *RVS167*, that is also required for viability upon starvation and for correct actin localization [37]. An *rvs167* gene disruption mutant has the same endocytic defect as the *end6-1* mutant and the *rvs161* disruption mutant [34]. *VRP1* encodes a protein called verprolin [38]. Verprolin is required, like Rvs161p and Rvs167p, for correct actin cytoskeleton localization. It was named verprolin because it contains a very high percentage of proline residues.

Both the *end3-1* [39] and *end4-1* mutations affect actin localization, and *end4-1* is allelic to *sla2* (*s*ynthetic *l*ethal with *a*bp1), a mutation identified by its lethality in conjunction with disruption of the *a*ctin *b*inding *p*rotein gene *ABP1* (ref. 40). End3p has an EH domain [39, 41], which was first found in the Eps15 protein, a

putative target of the epidermal growth factor receptor kinase. Eps15 may also function in endocytosis, because it binds to the clathrin adaptor complex, HA-2 [42]. Clathrin heavy chain and light chain somehow facilitate the internalization step of endocytosis in yeast, because mutants in these two genes show reduced kinetics of α-factor internalization [24, 43, 44, see below].

It is clear from the discussion above that our screen for *end* mutants identified several components or regulators of the actin cytoskeleton. We can conclude that the actin cytoskeleton plays a central role in the internalization stage of endocytosis in yeast. Not all mutations that disturb the actin cytoskeleton, however, affect endocytosis. Major rearrangements in the cytoskeleton caused by loss of the actin-binding proteins profilin or tropomyosin 1, or mutation of the *MYO2* gene encoding a type V myosin, do not have major effects on the kinetics of α-factor internalization [34].

A synthetic lethal approach for isolation of *end* mutants has also been developed in our laboratory [45]. Yeast strains deficient in vacuolar acidification due to deletion of the *VMA2* (*VAT2*) gene, which encodes a component of the vacuolar H^+-ATPase, are viable only on media with a pH near 5. It was hypothesized that endocytosis may allow sufficient acidification of the vacuolar system in these strains to allow for growth on low pH medium in the absence of vacuolar H^+-ATPase activity [46]. This supposition is supported by the finding that both the *end3* and *end4* deletion are lethal in combination with the *vma2* mutation, even on acidic medium at a temperature permissive for the growth of *end3* and *end4* single mutant strains [45]. This synthetic lethality was used to develop a new screen for *end* mutants. A strain harbouring both a chromosomal deletion of *VMA2* and a *URA3*-marked *VMA2* plasmid was mutagenized, and the cells were screened for their ability to lose the *VMA2* plasmid. Cells which cannot lose the plasmid remain Ura^+ and are inviable on medium containing 5-fluoroorotic acid, which selects against Ura^+ cells. All of the strong mutants obtained in this screen either do not have a visible vacuole or possess a vacuole but are deficient in fluid-phase endocytosis as revealed by an inability to accumulate LY in the vacuole. Stage-specific assays showed that out of seven mutants, four are defective in the internalization step of endocytosis and three are defective in transport of internalized material to the vacuole (as revealed by a defect in vacuolar degradation of the internalized α-factor). The mutants defective for internalization are *end8-1*, *end9-1*, *end10-1* and *end11-1*, and the mutants defective for delivery of endocytic content to the vacuole are *end12-1*, *end12-2* and *end13-1*. In each case, except *end11-1*, the Ts^- growth defect cosegregates with the *end* mutation through several outcrosses. Cells carrying *end11-1* grow poorly at 24 and 37 °C [45]. Recent results indicate that the *end8-1* and *end11-1* mutants are affected in lipid biosynthesis (A.M., unpublished results).

The three mutants defective in later stages of the endocytic pathway are compromised in their ability to deliver newly synthesized soluble proteins to the vacuole. Since the delivery of newly synthesized vacuolar proteins from the Golgi to the vacuole occurs via a late endocytic compartment(s), such a pleiotropic effect was expected. Complementation analysis with a representative of each previously isolated *vps* (*v*acuolar *p*rotein *s*orting) complementation group demonstrated that *end12-1* and *end12-2* are allelic to *vps34*, and *end13-1* is allelic to *vps4* [47, 48]. *VPS34* encodes a phosphotidylinositol-3 kinase (PI-3 kinase) that has been postulated to play a role in sorting events in a late Golgi compartment [49]. Our data suggest that PI-3 kinase activity is also required for endosomal function in yeast. Vps4p is an ATPase of the CDC48/PAS1/SEC18 family (GenBank U25842x3).

Another screen for endocytosis mutants was developed based on the downregulation of the a-factor receptor. The a-factor receptor is expressed from a controllable GAL promotor. When receptor synthesis is shut off over time, the cells lose the ability to mate. Several *ren* (*r*eceptor *en*docytosis) mutants were isolated that retain mating ability for longer periods of time, suggesting a defect in receptor internalization or an alteration of its intracellular trafficking so that the receptor recycles back to the cell surface rather than gets degraded in the vacuole. One of the mutant genes, *ren1*, is allelic to *VPS2* [23].

GTPases function at postinternalization steps in the yeast endocytic pathway. Ypt51, 52 and 53 are homologous to the mammalian rab5 protein, and mutants in these Ypt proteins affect an early postinternalization step of the endocytic pathway in yeast [50, 51]. Ypt7p is homologous to mammalian rab7 and functions at a late stage of the endocytic pathway [52, 53]. Deletion of a gene encoding a protein related to dynamin, *DNM1*, affects trafficking through endosomes [54].

Myosin and actin-dependent endocytosis

As described above, the initial analysis of genes required for endocytosis in yeast quickly revealed that the actin cytoskeleton plays a key role in this process. The involvement of actin, but not tubulin, in the internalization of the α-factor receptor was first demonstrated using conditional alleles of the *ACT1* and *TUB2* genes. A requirement for an actin-bundling protein homologous to the mammalian fimbrin (Sac6p) was also revealed [55].

What type of molecule could connect the cortical actin cytoskeleton to the plasma membrane and drive the formation the endocytic vesicles? Type I myosins constitute a family of unconventional myosins present in a

wide variety of cell types. These molecules show a characteristic N-terminal actin/ATP-dependent motor domain followed by a short C-terminal tail that can bind in vitro to acidic phospholipids. Their biochemical characteristics and subcellular localization to membranes suggest a role in processes that involve membrane dynamics such as organelle and vesicle movement, phagocytosis, pinocytosis and cell motility [56].

Calmodulin plays a key role in unconventional myosin function and is also required for the internalization step of endocytosis in yeast [57]. Two genes encode type I myosins in yeast: *MYO3* and *MYO5* [58, 59]. Deletion of either *MYO3* or *MYO5* does not lead to any obvious phenotype for growth. However, absence of both proteins in the same cell compromises growth almost completely. In order to analyse the function of type I myosins in endocytosis, a temperature-sensitive allele of *MYO5* was created (*myo5-1*). The double-deleted mutant (*myo3Δ*, *myo5Δ*) with the *myo5-1* allele on a plasmid shows about a 50% reduction in the rate of α-factor uptake at permissive temperature. Internalization is further reduced to 6% of the wild-type rate when the cells are shifted to restrictive temperature. Fluid-phase endocytosis, as measured by LY uptake, is also defective (M.I.G., unpublished data). In contrast, biosynthetic membrane traffic to the vacuole or to the plasma membrane remains unaffected [59]. These results demonstrate a direct role of a type I myosin in fluid-phase and receptor-mediated endocytosis in yeast.

The involvement of type I myosins in endocytosis is not a specific feature of yeast. Two recent studies [60, 61] show that type I myosins are also involved in endocytosis in *D. discoideum*. Five different myosin I proteins have been described in these cells (*MYOIA-F*) [62]. Among these, *MYOIB* shows maximal homology to the yeast type I myosins. Deletion of single myosin I genes leads to rather mild defects in bacteria phagocytosis and fluorescein isothiocyanate (FITC)-dextran pinocytosis [60–63]. However, when null mutations of *MYOIB* are combined with null mutants of *MYOIA* or *IC*, a synergistic defect in pinocytosis (up to 60% defective) is observed, whereas the phagocytic defect remains the same [60]. The endocytosis of a membrane protein is also severely impaired in the double mutants. Additionally, deletion of *MYOIB* in combination with *MYOIC* or *MYOIC* and *MYOID* leads to a synergistic reduction in pinocytosis, whereas the phagocytic defects of *MYOIB* and *MYO1C* are not additive [61]. Traffic through the endocytic recycling pathway is normal in these mutants.

Are actin/myosin and clathrin-dependent endocytic pathways independent? The contribution of clathrin to fluid-phase and receptor-mediated endocytosis in yeast is not well understood. Cells lacking clathrin heavy chain only show a 50% reduction in the uptake of α-factor and pheromone receptors [24, 44]. Several possibilities exist to explain this partial inhibition. First, clathrin may only participate in a subset of internalization events in yeast as in animal cells. Second, clathrin could participate in all internalization events, but not be absolutely essential mechanistically. Third, the clathrin requirement may be indirect. If clathrin participates directly at the internalization step, actin and myosin are probably also required for clathrin-dependent endocytosis, because they seem to affect all endocytic internalization events in yeast thus far examined. Interestingly, the genes encoding two of the isolated *end* mutants (*end6* and *end3*) that are defective for actin organization show homology to mammalian proteins (amphiphysin and eps15, respectively) that have been shown to interact with the clathrin/dynamin machinery [42, 64].

The situation is apparently similar in *D. discoideum* even though the contribution of clathrin to the pinocytic pathway is clearly more important. Clathrin-coated pits have been observed by electron microscopy, and deletion of the clathrin heavy chain reduces the uptake of FITC-dextran four- to fivefold [65].

The quantitative contribution of clathrin to total fluid-phase endocytosis in mammalian cells seems to vary depending on the cell type and the environmental conditions, as does the requirement for an intact actin cytoskeleton [66–68]. Internalization of GPI-anchored proteins through caveolae in epithelial cells is completely blocked by cytochalasin D [12], but the contribution of caveolar internalization to total fluid-phase endocytosis under nonstimulated conditions has not been determined. Actin is required for both clathrin-dependent and -independent uptake in the apical, but not the basolateral, side of Madin Darby canine kidney (MDCK) cells [8, 69]. The pinocytic requirements at the epithelial apical surface may resemble those of yeast cells. It has been proposed that clathrin-dependent pinocytosis may require an actin/myosin mechanism to overcome forces generated by turgor pressure when cells are exposed to the external environment [70]. A result that supports this view is the suppression of the pinocytic defect in *D. discoideum* double myosin null mutants when the amoebas are allowed to attach to substrate [60]. It is important to note that morphological and biochemical data suggest interactions between clathrin coats and the actin cytoskeleton [71, 72].

The mechanism by which actin and myosin drive the formation of pinocytic vesicles is still completely unknown. It is possible that the same basic mechanism that has been proposed to function in phagocytosis and macropinocytosis also works in budding of uncoated micropinocytic vesicles. The signal transduction pathways that lead to activation of these two endocytic pathways are probably different, because *D. discoideum* mutants differentially affect them [73, 74], but the molecular machinery involved seems to be very similar. Both phagocytosis and macropinocytosis are blocked in

the presence of cytochalasin D [75, 76], and the formation of the phagocytic cups as well as membrane ruffles and macropinocytic processes are associated with directed actin polymerization, and triggered by the activation of GTPases [76–78]. In addition to actin, a subset of actin-binding proteins, including type I myosins, associate transiently with the phagocytic cup of both amoebas (coronin, a 30-kD actin-bundling protein and myosin I) and macrophages (talin, coronin and myosin I) [75, 78–81]. In a careful electron microscopy analysis of *Acanthamoeba* type I myosins, the phosphorylated form of MyoIB and MyoIC were shown to accumulate 5- and 10-fold over invaginations and protrusions of the plasma membrane and phagocytic cups, respectively. These data suggest an important role of phosphorylation in the regulation of these proteins [79].

It has been proposed that localized actin polymerization can cause deformation of the plasma membrane and therefore generation of phagocytic and pinocytic projections [82]. Type I myosins could participate in this process or perhaps function in the detachment of the endocytic vesicle from the plasma membrane. Consistent with this latter function is the observation that amoebas with multiple myosin I null mutations do not present a quiescent plasma membrane as one might expect if the type I myosins were required for membrane deformation. On the contrary, these mutants accumulate surface projections (pseudopodia and crowns) associated with circular rings and projections of the actin cytoskeleton [60, 61]. Therefore, we think it is more likely that type I myosins act at a late stage of vesicle formation.

Ubiquitin-dependent internalization of plasma membrane proteins

Several different signals mediate the entry of plasma membrane proteins into the endocytic pathway. These sequences reside in the cytoplasmic domains of endocytosed proteins, and are based on tyrosine or di-leucine motifs [83]. Recently, a novel signal has been added to this list. The modification of the cytoplasmic domains of proteins with ubiquitin has been shown to trigger the internalization of cell surface proteins.

Ubiquitin is a 76-amino acid polypeptide that is probably ubiquitous in expression among eukaryotic cells, though it has not yet been detected in prokaryotes. It becomes conjugated to a wide variety of proteins that reside in the cytosol and in the membranes and lumen of organelles. Ubiquitin is joined to proteins via an isopeptide bond linking the C-terminal ubiquitin glycine to the ε-amino group of lysine residues within the modified protein. This process requires the action of two or three enzymes. A ubiquitin-activating enzyme (E1) first forms a high-energy thioester bond to ubiquitin in an ATP-dependent reaction. Ubiquitin is then transferred to a target protein by the action of ubiquitin-conjugating enzymes (E2s), which, in some cases, work together with a ubiquitin protein ligase (E3) [84].

Until recently, the only known function of ubiquitination was to target modified proteins for degradation by a multisubunit cytosolic protease, the 26S proteasome. It was known, however, that a number of membrane proteins also become modified with ubiquitin, and the role of ubiquitination in these cases was less clear [84]. In animal cells, plasma membrane receptors become ubiquitinated in response to stimulation by ligand, and these proteins include the T-cell receptor, the FcεRI receptor and the receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), c-kit and growth hormone [85–91].

The first clue that led to defining a role for plasma membrane protein ubiquitination came from studies in yeast on the peptide transporter, Ste6p. Ste6p spends at least part of its life on the plasma membrane, and from there it is constitutively endocytosed and transported to the vacuole, where it is degraded. In an *end4* mutant that cannot endocytose, the protein accumulates in the plasma membrane in ubiquitinated forms. Furthermore, degradation of Ste6p, which occurs in the vacuole, was inhibited in mutants that lack ubiquitin-conjugating enzymes [28, 29]. Together, these observations hinted that the ubiquitination of Ste6p at the plasma membrane somehow mediates its degradation in the vacuole.

Experiments performed with another plasma membrane protein from yeast proved that ubiquitination of plasma membrane proteins leads to their degradation in the vacuole by serving as a signal for internalization into the endocytic pathway. The protein analysed in these studies was the G protein-coupled receptor, Ste2p. In the absence of ligand, Ste2p is constitutively internalized at a slow rate. Internalized receptor does not recycle to the plasma membrane, but instead is transported through endosomal compartments to the vacuole, where it is degraded. Binding of α-factor to Ste2p stimulates a signal transduction pathway that leads to changes in gene expression and cell morphology necessary for mating, and stimulates internalization of the receptor 5- to 10-fold [92]. Pheromone binding also induces modifications of the receptor cytoplasmic tail. The tail becomes hyperphosphorylated on serine and threonine residues and is modified with ubiquitin on many of the tail's eight lysines [93, 94].

The ubiquitination of cytosolic proteins results in their degradation by the proteasome; however, mutants that carry defective proteolytic subunits of the proteasome degrade cell surface Ste2p with the same kinetics as wild-type cells. In contrast, mutants that lack active vacuolar hydrolases do not degrade the receptor even long after exposure to α-factor. Therefore, the ubiquitination of Ste2p does not function to target it for degradation by the proteasome.

To determine the function of Ste2p ubiquitination, the behaviour of the Ste2p-α-factor complex was analysed in mutants that lack ubiquitin-conjugating enzymes. Ten enzymes, encoded by *UBC* genes, have been identified in yeast, and mutants that lack multiple enzymes of the Ubc1/Ubc4/Ubc5 family are defective in their ability to internalize radiolabelled α-factor. The *ubc4ubc5* double mutant internalizes α-factor 15-fold slower than do wild-type cells and does not efficiently ubiquitinate Ste2p in response to α-factor binding, suggesting that ubiquitination of the α-factor receptor itself is required for its internalization [94].

The ubiquitination of Ste2p, as of all proteins, occurs on lysine residues. Therefore, further support for the hypothesis that receptor ubiquitination triggers internalization came from studies that identified a lysine-dependent internalization signal within the Ste2p cytoplasmic tail. The wild-type tail carries multiple, redundant internalization signals. One of these signals has been defined, the nine amino acid sequence SINNDAKSS, and is necessary and sufficient for the internalization of a severely truncated version of the receptor. The serines and aspartate residues within this sequence affect its efficacy, but the only single amino acid essential for internalization is the lysine [95]. Not only is the SINNDAKSS lysine essential for internalization of truncated receptor, but it is also essential for its ubiquitination [94]. Together these experiments demonstrate that the ubiquitination of Ste2p, mediated by the Ubc4 and Ubc5 conjugating enzymes, occurs in response to α-factor binding and is required for the ligand-stimulated internalization of the receptor-ligand complex.

The cytoplasmic tail of Ste2p is phosphorylated at a basal level and becomes hyperphosphorylated in response to pheromone binding. Other G protein-coupled receptors are also phosphorylated, and in some cases this modification is necessary for downregulation of the receptor. The function of Ste2p tail phosphorylation is not well understood; however, recently it has been shown that the phosphorylation of at least several serines is required for internalization of truncated receptor. Analysis of mutations in the SINNDAKSS sequence indicate that phosphorylation of one or more of the three serines is essential for internalization mediated by this signal, and that phosphorylation probably mediates internalization by positively regulating ubiquitination at the neighboring lysine. Mutation of the three SINNDAKSS serines to alanines abolishes both ubiquitination and internalization of truncated receptor [94]. Furthermore, though six other serines remain in the truncated receptor tail, the AINNDAKAA mutant was neither phosphorylated at a basal level in the absence of ligand nor hyperphosphorylated in response to α-factor binding (L.H. and B. Zanolari, unpublished data). These observations led to the model that pheromone binding stimulates receptor internalization by inducing a conformational change that leads to hyperphosphorylation of specific receptor tail serines. The phosphorylation of these serines is necessary to positively regulate ubiquitination at neighbouring lysine residues, though ubiquitination may also be facilitated by the ligand-induced conformational change itself. An increase in the rate of receptor ubiquitination then leads to an increased rate of internalization by an as yet undefined mechanism.

The ubiquitination of cytosolic proteins, such as the cyclins and the transcription factor inhibitor IκB, which undergo regulated ubiquitination and degradation, also requires serine phosphorylation to regulate ubiquitin conjugation [96–98]. Therefore, it appears that phosphorylation is a general mechanism for promoting ubiquitination at regulated sites. It is not clear if the only role of Ste2p phosphorylation is to facilitate ubiquitination, or whether phosphorylated residues may participate in signal transduction or receptor downregulation via pathways other than endocytosis.

The internalization of plasma membrane proteins through a ubiquitin-dependent process is a general phenomenon in yeast. As mentioned above a number of other plasma membrane proteins are endocytosed and degraded in the vacuole. In many cases these proteins have been shown to accumulate in ubiquitinated forms at the plasma membrane when endocytosis is blocked in an *end4* mutant, and their degradation is inhibited in mutants that lack either ubiquitin-conjugating enzymes or the Rsp5 ubiquitin protein ligase [99–101]. Some of these proteins undergo both constitutive and stimulated internalization/degradation, and some are internalized only constitutively, indicating that ubiquitin probably mediates both types of internalization. The correlation between the list of yeast plasma membrane proteins known to undergo endocytosis and those that become modified with ubiquitin is excellent.

Mammalian receptors are also endocytosed by a ubiquitin-dependent mechanism. The EGF receptor, the PDGF receptor, the c-kit receptor and the growth hormone receptor all undergo stimulated internalization and lysosomal degradation in response to ligand binding. These receptors also become modified with ubiquitin in response to stimulation with ligand (see references above). Recently, mammalian receptor internalization and lysosomal degradation have been linked to the ubiquitin-conjugating system. Wild-type Chinese hamster ovary (CHO) cells internalize and degrade transfected growth hormone receptor in the lysosome after being exposed to growth hormone. In contrast, a CHO Ts^- mutant defective in the ubiquitin-activating enzyme (E1) is unable to degrade and internalize transfected growth hormone receptor at the nonpermissive temperature [91].

Though it appears that mammalian cells employ ubiquitin as an endocytosis internalization signal, it is unclear

how ubiquitination fits in with other internalization signals that have been characterized. The tyrosine-based signal mediates the internalization of proteins that are constitutively endocytosed and recycled back to the plasma membrane. Ubiquitin, however, seems to act as a signal that triggers downregulation of receptors stimulated by ligand binding. Internalization of stimulated growth factor receptors usually leads to transport to and degradation in the lysosome, and ubiquitin may indicate this fate, signalling degradation rather than return transport to the plasma membrane. Ubiquitin may act together with a tyrosine-based signal as the EGF receptor requires tyrosine-based sequences for internalization and subsequent degradation [102]. It remains to be determined how ubiquitination promotes receptor internalization and how this stimulated internalization signal differs in action from previously defined constitutive internalization signals.

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