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Actin and fimbrin are required for the internalization step of endocytosis in yeast

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In *Saccharomyces cerevisiae*, α-factor is internalized by receptor-mediated endocytosis and transported via vesicular intermediates to the vacuole where the pheromone is degraded. Using β-tubulin and actin mutant strains, we showed that actin plays a direct role in receptor-mediated internalization of α-factor, but is not necessary for transport from the endocytic intermediates to the vacuole. β-tubulin mutant strains showed no defect in these processes. In addition, cells lacking the actin-binding protein, Sac6p, which is the yeast fimbrin homologue, are defective for internalization of α-factor suggesting that actin filament bundling might be required for this step. The actin dependence of endocytosis shows some interesting similarities to endocytosis from the apical membrane in polarized mammalian cells.

Key words: α-factor/cytoskeleton/receptor-mediated endocytosis/*Saccharomyces cerevisiae*/tubulin

Introduction

The endocytic pathway has been studied for many years in mammalian cells, originally by means of a morphological description that led to the identification of coated pits, coated vesicles and different endocytic compartments (Goldstein *et al.*, 1985). Biochemical studies have focused mainly on the role of clathrin and its associated proteins, adaptins, in the internalization step (Pearse and Robinson, 1990), and more recently on the role of rab proteins in endocytic vesicular traffic (Bucci *et al.*, 1992). Unfortunately, due to limitations inherent to the system, it is often difficult to obtain mutant cells that are defective in particular proteins in order to test for their functions *in vivo*. For this reason, despite over a decade of work by many laboratories, the role of clathrin heavy and light chains in animal cells is still unclear. It is not known whether clathrin polymerization is a driving force for endocytosis nor to what extent clathrin is necessary for protein sorting.

In order to test for the *in vivo* functions of proteins in endocytosis and to identify novel components of the endocytic machinery the yeast *Saccharomyces cerevisiae*, presents certain advantages, foremost of which are the powerful genetic techniques that are available and the facile growth of large quantities of cells expressing particular phenotypes or proteins. For example, the clathrin heavy chain was deleted from yeast and in this manner it was proven that clathrin is dispensable for secretion (Payne and Schekman, 1985), but plays a role in retention of the Golgi enzymes (Seeger and Payne, 1992a), transport of soluble vacuolar hydrolases to the vacuole (Seeger and Payne, 1992b) and receptor-mediated endocytosis (Payne *et al.*, 1988). Genetic techniques have also been used to study various cellular processes and structures, including secretion (Pryer *et al.*, 1992), the cell cycle (Cross *et al.*, 1989) and signal transduction (Marsh *et al.*, 1991). Particularly relevant to this study is the detailed genetic analysis of the yeast cytoskeleton (Huffaker *et al.*, 1987). A large number of mutations in the genes encoding the major proteins of the yeast cytoskeleton, *TUB1* (one of two genes encoding α-tubulin), *TUB2* (encoding β-tubulin) and *ACT1* (actin) were obtained and have been helpful in describing the functions of the cytoskeleton. In addition, mutants in other cytoskeletal proteins are available (Brown, 1993).

The advantages of this large pool of mutants and the ease of isolation of novel ones makes yeast an excellent system to study endocytosis. Two markers have been developed to study endocytosis in yeast, lucifer yellow CH to follow fluid phase endocytosis and α-factor to follow receptor-mediated endocytosis (Riezman, 1985; Chvatchko *et al.*, 1986; Jenness and Spatrick, 1986). α-factor binds specifically to its receptor, the product of the *STE2* gene, which is a G protein-coupled receptor (Jenness *et al.*, 1983; Miyajima *et al.*, 1987; Whiteway *et al.*, 1989), and triggers its endocytosis (Jenness and Spatrick, 1986; Zanolari *et al.*, 1992). Endocytosis of the α-factor receptor is independent of pheromone-specific signal transduction (Zanolari *et al.*, 1992). α-factor is particularly useful as an endocytic marker because distinct steps of the endocytic pathway, such as internalization, passage through endocytic intermediates and delivery to the vacuole can be differentiated (Singer and Riezman, 1990; Dulic *et al.*, 1991). Recently, two genes that are essential for the internalization step of endocytosis were isolated (Raths *et al.*, 1993).

In order to study the role of the cytoskeleton in endocytosis we measured endocytosis in strains carrying conditional mutations in actin (Novick and Botstein, 1985), β-tubulin (Huffaker *et al.*, 1988) and carrying deletions in the genes encoding the actin-binding protein Abp1p and the yeast homologue of fimbrin, Sac6p (Drubin *et al.*, 1988; Adams and Botstein, 1989). These studies showed that a functional actin cytoskeleton, but not the microtubule network, is essential for the internalization step of endocytosis. No apparent requirement for either cytoskeletal network was seen for subsequent steps of the endocytic pathway.

Results

Actin mutants are defective in endocytosis

Two different conditional mutations each for actin and β-tubulin were tested for their affect on the accumulation of lucifer yellow CH (LY) in the vacuole, an assay for fluid phase endocytosis (Riezman, 1985). The temperature-sensitive actin mutant strains, *act1-1* (strain EK14-5D) and

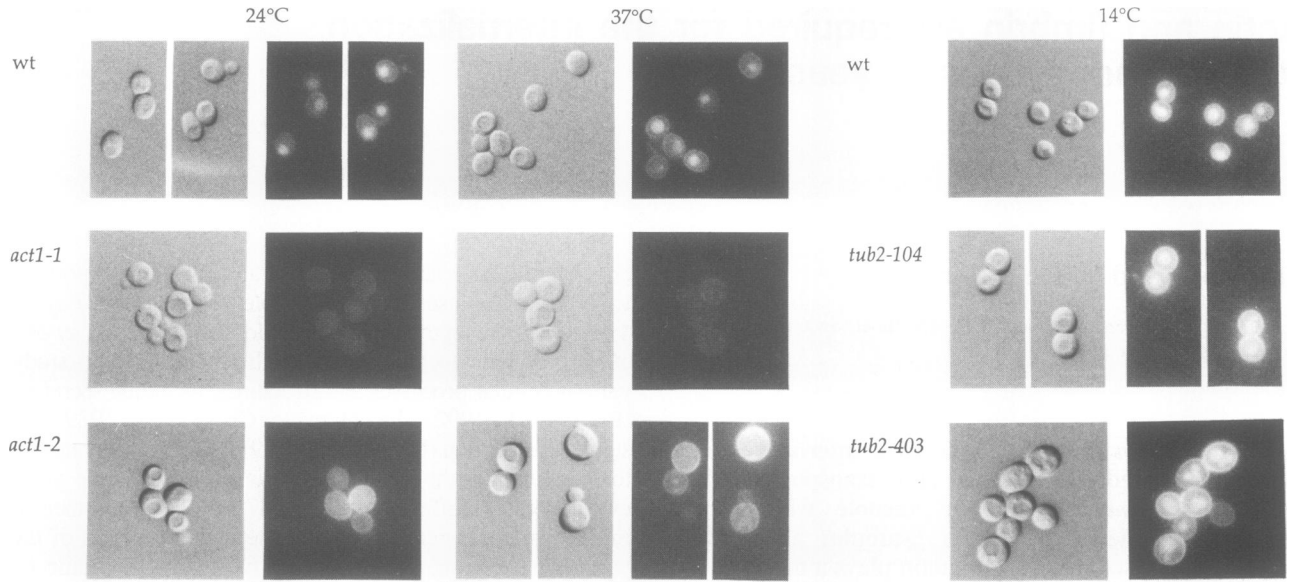

Fig. 1. Accumulation of LY by actin and tubulin mutants. After harvest, one-half of the actin mutant cells and the wild-type cells were incubated at 24°C, the other half at 37°C, both for 1 h in the presence of 4 mg/ml LY. The β-tubulin mutants and wild-type cells were preincubated for 20 h at 14°C before incubation with 4 mg/ml LY for 3 h at 14°C. All cells were washed and mounted as described in Materials and methods. Cells were visualized using Nomarski (left-hand columns) and fluorescence optics (right-hand columns).

act1-2 (strain EK15-2B) which show an aberrant cytoskeleton at the non-permissive temperature (37°C) and to a lesser extent at permissive temperature (24°C) (Novick and Botstein, 1985) and wild-type cells (RH144-3D), were grown at 24°C to mid-log phase, washed and incubated with LY for 1 h at 24 or 37°C. After thorough washing the cells were visualized by fluorescence and Nomarski optics. Both mutant strains showed substantially reduced accumulation of LY in the vacuole at either temperature when compared with wild-type cells (Figure 1). In marked contrast, the cold-sensitive β-tubulin strains, *tub2-104* (strain EK5-1B) and *tub2-403* (strain EK6-2C), which were preincubated for 20 h at the restrictive temperature of 14°C followed by a 3 h incubation with LY at the same temperature, accumulated LY normally in the vacuole. Under these incubation conditions no microtubules could be seen by immunofluorescence and the cells were arrested in the cell division cycle (Huffaker *et al.*, 1988). These results suggest that either internalization of LY and/or its transport to the vacuole is dependent on a functional actin cytoskeleton, but not on cytoplasmic microtubules. In order to differentiate between the internalization and subsequent transport steps to the vacuole, we analysed these steps separately following the receptor-mediated endocytosis of α-factor (Dulic *et al.*, 1991). Log phase cells grown at 24°C of the *act1-1* and *act1-2* mutant strains were washed and [^{35}S]α-factor was bound at 0°C. The cells were then centrifuged and resuspended in prewarmed 24 or 37°C medium. After various incubation times at the restrictive temperature, samples were taken and treated with pH 6 buffer to remove unbound α-factor or with pH 1 buffer to remove unbound α-factor and α-factor bound to receptors at the cell surface. The [^{35}S]α-factor was quantified and the ratio of c.p.m. at pH 1 to c.p.m. at pH 6 was used to calculate the percentage of α-factor internalized. α-factor uptake was partially defective at 24°C and was completely blocked in *act1-1* cells at the restrictive temperature (Figure 2c) compared with a wild-type strain treated with the same protocol (Figure 2a). The partial α-factor uptake at 24°C is consistent with the partial disruption of actin function at this temperature (Novick and Botstein, 1985). *Act1-2* cells showed a stronger uptake defect at 24°C than *act1-1* cells, but were not completely defective at 37°C (Figure 2d). Both β-tubulin mutant strains were grown at 30°C to mid-log phase, washed and preincubated in prewarmed 30°C medium or in precooled 11°C for 20 h to ensure the mutant block. The cells were then centrifuged, resuspended in precooled 11°C or prewarmed 30°C medium, radioactive α-factor was added and further incubated for a period of 1 h at 30°C or 2 h at 11°C. As seen in Figure 2b, the rate and the amount of α-factor internalization by the β-tubulin mutants were indistinguishable from wild-type control cells treated in the same manner.

The block in endocytosis in the actin mutants is direct

The rapidity of the block of α-factor uptake in the *act1-1* mutant (Figure 2c) strongly suggests that actin plays a direct role in the internalization step of endocytosis. However, it has been shown that many yeast secretion mutants block accumulation of LY in the vacuole (Riezman, 1985) and it has also been shown that the *act1-1* mutant has a partial defect late in the secretory pathway, accumulating an abnormally large pool of invertase in secretory vesicles under non-permissive conditions (Novick and Botstein, 1985). Therefore, one could argue that the requirement for actin in endocytosis is indirect, resulting from the defect in secretion. To exclude this possibility we measured α-factor internalization in *sec10* mutant cells that also accumulate secretory vesicles at the non-permissive temperature (Novick *et al.*, 1980). *Sec10* cells (RH1536) were grown overnight at 24°C, washed and preincubated for 20 min at the permissive or non-permissive temperature before addition of [^{35}S]α-factor. Internalization of α-factor was then measured as a function of time. The results for *sec10* mutant cells and wild-type cells treated by the same protocol were

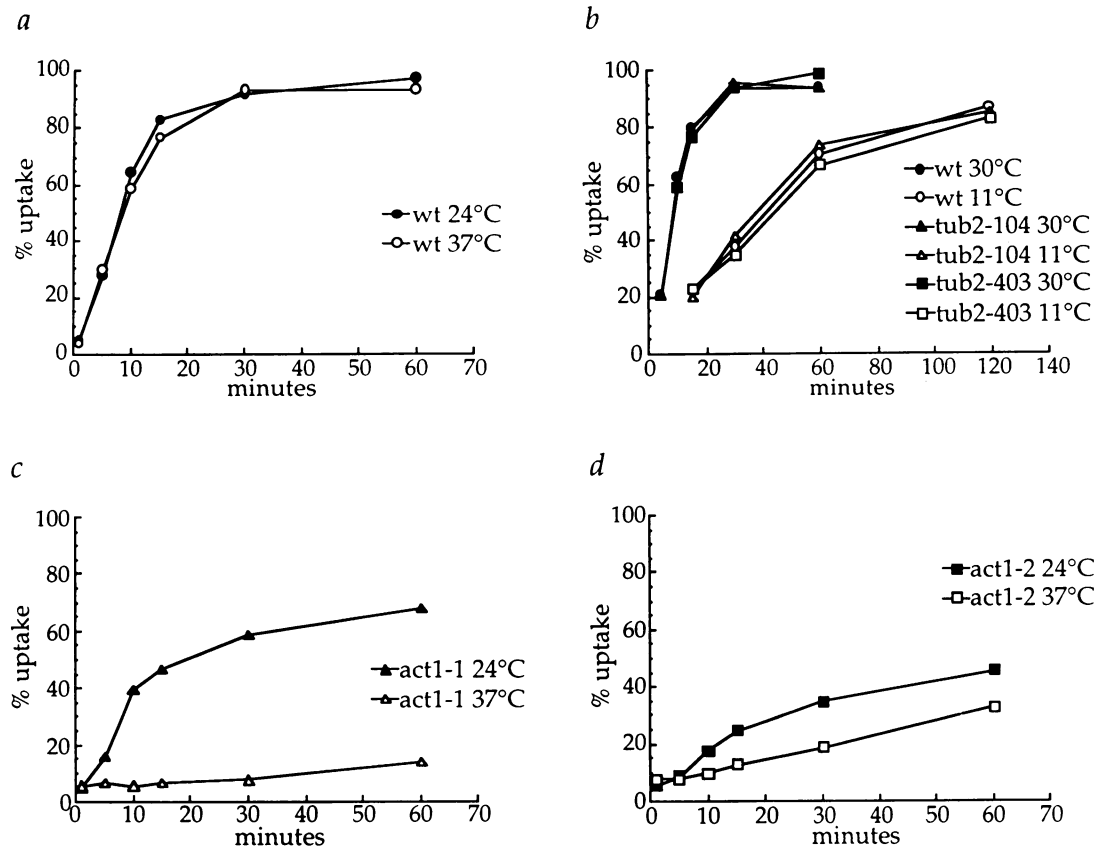

Fig. 2. Internalization of [^{35}S]α-factor by wild-type (**a**), β-tubulin mutant (**b**), *act1-1* (**c**) and *act1-2* cells (**d**). After binding of [^{35}S]α-factor on ice, the wild-type cells (a) and the actin mutant cells (c and d) were resuspended in prewarmed 24 or 37°C medium. The β-tubulin mutant and wild-type control cells (b) were preincubated at 11 or 30°C for 20 h, resuspended in medium of the same temperature and [^{35}S]α-factor was added. At the times indicated, samples were diluted to either ice-cold 100 mM potassium phosphate buffer (pH 6) or ice-cold 50 mM sodium citrate buffer (pH 1) and filtered to determine the extent of α-factor internalization.

identical (Figure 3). This result, along with the fact that the block in internalization of α-factor in the *act1-1* mutant is immediate and complete upon shift to 37°C, strongly suggests that actin is directly involved in the internalization step of endocytosis.

Actin is apparently not required for traffic from endosomes to the vacuole

Upon internalization of α-factor in wild-type cells, the pheromone is targeted through two intermediate compartments (endosomes) to the vacuole, where it is finally degraded by resident proteases (Singer and Riezman, 1990). In order to determine whether actin is involved in these later stages of endocytosis, *act1-1* and wild-type cells were grown to mid-log phase, washed and incubated at 15°C with α-factor for 25 min. This treatment allows internalization and accumulation of the pheromone in the endocytic intermediate compartments because at 15°C, further transport to the vacuole is differentially slowed down (Singer and Riezman, 1990). The cells were then collected by centrifugation and resuspended in 37°C medium, samples were taken at various times and washed in pH 1 and pH 6 buffers. The radioactivity was extracted from the cells and analysed on TLC plates (Dulic *et al.*, 1991). Transport of α-factor to the vacuole was monitored by the disappearance of intact α-factor and by the appearance of degradation products (Figure 4). In both wild-type and *act1-1* mutant cells, the internalized α-factor (pH 1-resistant) was degraded. This process was only

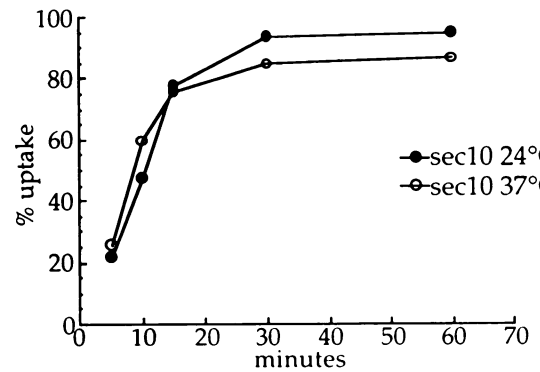

Fig. 3. Internalization of [^{35}S]α-factor by *sec10* mutant cells. After harvest, *sec10* mutant cells were preincubated in prewarmed 37°C medium and radioactive α-factor was added. At the indicated times, samples were taken and processed as described in the legend to Figure 2.

slightly slower in the *act1-1* mutant than in wild-type cells. The difference in the intensity of degradation products between the wild-type and the *act1-1* mutant resulted because wild-type cells continued to internalize and degrade α-factor after the shift to 37°C, whereas *act1-1* cells were immediately blocked in this process as seen by the presence of intact α-factor after pH 6 wash, but not pH 1 wash. This result suggests that actin is not required for transport from endosomes to the vacuole. No differences in α-factor transport to the vacuole were observed between wild-type

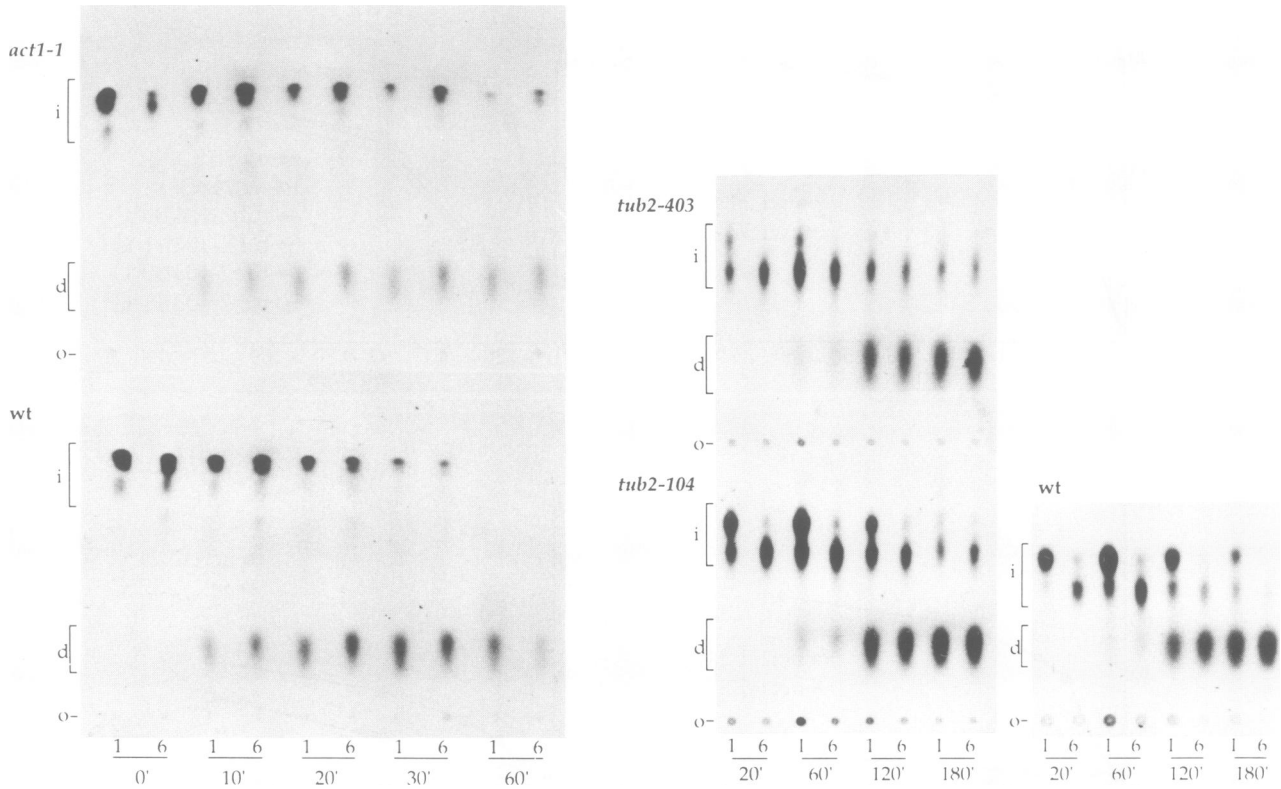

Fig. 4. Degradation of α-factor by *act1-1*, *tub2-104*, *tub2-403* and wild-type control cells. After harvest, *act1-1* and wild-type cells were allowed to bind radioactive α-factor for 1 h on ice. The cells were then pelleted at 4°C, resuspended in 15°C medium, and incubated for a further 25 min at 15°C to accumulate α-factor in the intermediate compartments. The cells were then pelleted again at 4°C and resuspended in 37°C medium. At the times indicated after the 37°C shift, aliquots were taken and diluted into pH 1 and pH 6 buffer, as described for the measurement of internalization in the legend to Figure 2. α-factor degradation (indicating delivery to the vacuole) was assessed by extracting the cell-associated radioactivity (see Materials and methods) and developing these samples on preparative TLC plates for subsequent fluorography. *Tub2-104*, *tub2-403* and wild-type control cells were grown at 30°C, centrifuged, resuspended in 14°C medium and preincubated for 20 h. The cells were then pelleted, resuspended again in 14°C medium and radioactive α-factor was added. At the indicated times, samples were taken and processed as described above. 1 and 6 denote samples diluted in pH 1 buffer or pH 6 buffer respectively. The positions of intact (i) and degraded (d) α-factor and the origin of migration (o) are indicated.

cells and the two β-tubulin mutants in a degradation assay performed at 14°C (Figure 4), confirming the results with LY that suggested that tubulin is not required for endocytic traffic to the vacuole.

Fimbrin is required for endocytosis

Proper actin function in numerous cellular processes depends on its regulated interaction with other proteins and its regulated formation of supramolecular structures. To test whether the actin function in endocytosis requires specific actin-binding proteins, we assayed two mutants with deleted genes encoding actin-binding proteins. One protein, Sac6p, was identified as a protein that binds to a yeast F-actin column (Drubin *et al.*, 1988) and in parallel, a mutation in its gene was found to suppress the *act1-1* temperature-sensitive growth defect (Adams and Botstein, 1989). The sequence of this gene revealed a protein that is the yeast homologue of mammalian fimbrin. Consistent with this finding is the fact that purified Sac6p bundles yeast actin fibres *in vitro* (Adams *et al.*, 1991). A yeast strain containing a disrupted *sac6* gene is viable at 24°C but not at 37°C and shows a disrupted actin cytoskeleton (Adams *et al.*, 1991). A *sac6* deletion mutant strain (EK30-1C) was grown overnight at 24°C and then assayed for α-factor uptake at 24°C after binding of [^{35}S]α-factor to cells at 0°C. α-factor internalization was clearly defective in the *sac6* mutant strain (Figure 5) providing evidence for a role of fimbrin in endocytosis.

Another actin binding protein, Abp1p, was identified by its binding to an F-actin column and localizes to cortical actin patches (Drubin *et al.*, 1988). The gene was subsequently cloned and a disruption allele was created that causes no apparent growth phenotype (Drubin *et al.*, 1990). An *abp1* deletion strain (RH2108) was grown and assayed as described for the *sac6* deletion strain but at 30°C. The *abp1* mutant strain showed no α-factor uptake defect (Figure 5).

Discussion

The main conclusion that can be drawn from this study is that the internalization step of the receptor-mediated endocytic pathway in yeast depends on a functional actin network but not on cytoplasmic microtubules. We obtained convincing and definitive results for a direct actin requirement using a strain carrying a conditional mutation in actin causing its rapid inactivation at the non-permissive temperature. In addition, we could show that the yeast fimbrin homologue Sac6p is required for endocytosis. The actin requirement that we saw for endocytosis cannot be due to the effect that actin mutants have on secretion. First, the endocytic defect in *act1-1* is immediate and complete as soon as the temperature is raised to 37°C. Second, the effect of

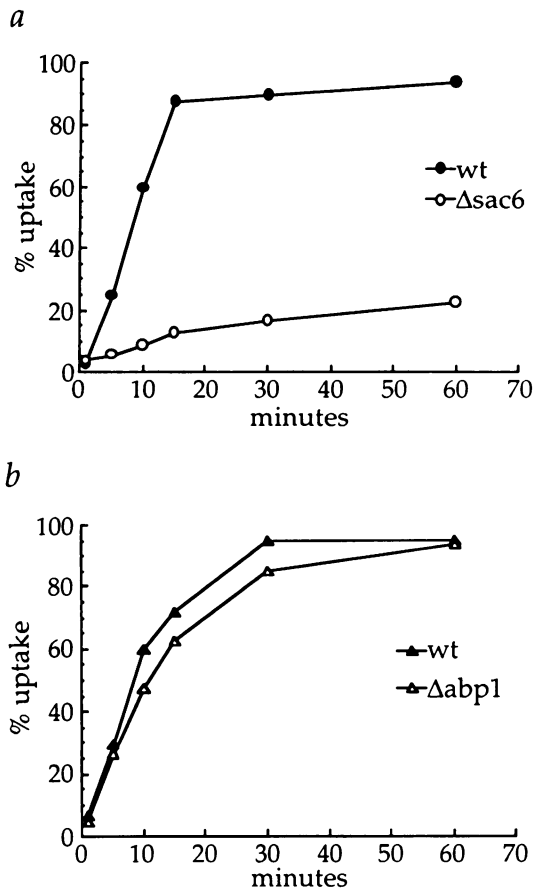

Fig. 5. Internalization of [^{35}S]α-factor by the *sac6* deletion mutant, the *abp1* deletion mutant and wild-type control cells. (**a**) The *sac6* deletion strain and the wild-type control strain were grown and assayed at 24°C as described for Figure 2a, c and d. (**b**) The *abp1* deletion strain and wild-type control strain were assayed in the same manner but at 30°C.

actin on total secretion is not very strong (Novick and Botstein, 1985). Third, a *sec10* mutation that had a very strong effect on invertase secretion and accumulated secretory vesicles (Novick *et al.*, 1980), had no effect on the internalization step of endocytosis even after a 20 min preincubation at 37°C.

We do not yet know how actin works on the molecular level during endocytic uptake nor how the mutated actin proteins are disrupted in their normal interactions. The failure of mutant *act1-1* protein to form visible cables *in vivo* suggests a defect in polymerization or bundling (Novick and Botstein, 1985). Our results with the *act1-2* mutant are also consistent with a role for actin polymerization and/or bundling. The fine actin cables seen in *act1-2* cells at the non-permissive temperature (Novick and Botstein, 1985), although disorganized, could reflect the partial ability of the mutant strain to polymerize actin and bundle actin filaments, thus allowing slow α-factor uptake. It is interesting to note that *sac6* was isolated as an allele-specific suppressor of *act1-1* (Adams and Botstein, 1989). Sac6p was shown to have 43% amino acid sequence homology with the vertebrate actin filament bundling protein fimbrin and was shown to bundle yeast actin *in vitro* (Adams *et al.*, 1991). In addition, immunofluorescence studies showed that in a yeast strain with a deleted *sac6* gene, only very few cells contained visible actin cables and these were drastically reduced in fluorescence intensity, supporting for the first time a role for fimbrin in bundling actin *in vivo*. The defect in endocytosis in a fimbrinless strain probably reflects the need for such an activity. We speculate that filamentous actin may have to be bundled into cables to constitute a strong enough structure to drive the scission of membrane invaginations from the surface. In this way, actin cables could build a network around membrane invaginations or form a lasso-like structure around their neck to pinch them off. A role for actin in scission of vesicles from the plasma membrane would be consistent with the phenotype found when MDCK cells were treated with cytochalasin D (Gottlieb *et al.*, 1993): the apical cell surface accumulated an increased number of elongated clathrin coated pits when treated with the drug.

The subcellular localization of Sac6p in yeast and fimbrin in animal cells, would be consistent with a direct role in endocytosis. Sac6p is found bound to actin filaments that traverse the cell, but also in the cortical actin patches (Drubin *et al.*, 1988). The structure and the function of the cortical actin patches are still unknown but an involvement in endocytosis can be envisaged as these actin structures are localized to the surface where endocytosis occurs. Fimbrin has been shown to be concentrated in microvilli and other cell surface structures in animal cells (Bretscher and Weber, 1980). Again, cytochalasin D inhibited endocytosis from the apical surface in MDCK cells where the microvilli are present and not from the basolateral surface (Gottlieb *et al.*, 1993). In addition to the cytochalasin D inhibition of endocytosis through apical clathrin coated pits, the drug has also been shown to inhibit the uptake of ricin, which is internalized via a clathrin-independent pathway by Vero cells (Sandvig *et al.*, 1989; Sandvig and van Deurs, 1990). The specificity of the effect of cytochalasin D is not clear, however, because colchicine treatment had a similar effect. In yeast, we do not know whether a clathrin-dependent and/or clathrin-independent pathway is used to internalize α-factor and LY, but a strain where the clathrin heavy chain was deleted shows a 50–65% decrease in its α-factor uptake rate (Payne *et al.*, 1988). As the cortical actin patches are spatially consistent with a role in endocytosis, we tested whether the actin-binding protein Abp1p, which exclusively binds to the cortical actin patches (Drubin *et al.*, 1988), is required for endocytosis. Cells lacking Abp1p were normal for α-factor uptake, suggesting that this protein is not required for endocytosis. This does not prove that Abp1p is not used in the endocytic pathway. As the function of Abp1p is not yet known, it is possible that other yeast proteins can perform the same or similar function and replace Abp1p in this pathway.

Another possibility for the mode of action of actin in endocytosis is that actin cables interact directly or indirectly with the plasma membrane and that invaginations arise by pulling the membrane along actin cables towards the centre of the cell. This would then involve an actin-binding motor molecule. A type I myosin would be a good candidate to act as a molecular motor since this molecule was shown to be able to bind membranes and to move actin filaments on a phospholipid substrate (Adams and Pollard, 1989; Zot *et al.*, 1992). Interestingly, dynamin, a microtubule-binding GTP hydrolysing motor protein (Shpetner and Vallee, 1989) has been shown to be encoded by the *shibire* gene, which is required for endocytosis in *Drosophila* (Collins, 1991). Therefore, dynamin binds microtubules *in vitro* and is

necessary for endocytosis, but a colocalization with microtubules has never been shown *in vivo*, nor has a requirement for microtubules been shown for the internalization step of receptor-mediated internalization. Interestingly, when *shibire* flies are incubated at the non-permissive temperature an increased number of elongated cell surface invaginations appear (Kosaka and Ikeda, 1983), reminiscent of the effect of cytochalasin D on the apical membrane of MDCK cells (Gottlieb *et al.*, 1993). Is it possible that dynamin can also work as an actin-based motor?

Recently, many new mutations in the actin gene were created by site-directed mutagenesis and each one was inserted into the yeast genome (Wertman *et al.*, 1992). Many of these mutants show different growth phenotypes suggesting defects in different cellular aspects. *In vitro* analysis of the mutant actins' ability to polymerize or to bind known actin-binding proteins, correlated to their endocytic phenotype *in vivo* could give us a further insight into the precise function of actin in the mechanism of the internalization step of endocytosis.

Another step during the process of endocytosis, the traffic from the endosomes to the vacuole, does not seem to be defective in the actin mutants. However, the experiments performed do not yet completely exclude an involvement of actin in this step. First, it could be that the *act1-1* allele is specifically inhibitory to uptake but is not inhibitory for the transport step. If this were the case, there must be mutant actin alleles that would specifically inhibit the transport step. Second, it is possible that *act1-1* cells have to be preincubated at the restrictive temperature to exhibit a defect in targeting α-factor from the endosomes to the vacuole. It is technically impossible to test the latter hypothesis, because uptake of α-factor is immediately blocked when cells are incubated at the non-permissive temperature.

Traffic from endosomes to the vacuole in yeast is also independent of cytoplasmic microtubules. The extended preincubation used with the cold-sensitive *tub2* mutant strains should have completely disassembled any residual microtubular structures. This finding contrasts with findings in animal cells where endosomal compartments are believed to associate with microtubules (Pierre *et al.*, 1992) and where colchicine slows down delivery of endocytosed markers to the lysosomes (Gruenberg and Howell, 1989). This is only a kinetic effect and it may be that endosome movement towards the centre of the cell, where late endosomes are located, is accelerated by attachment to microtubules. The spatial organization inside a yeast cell is different and therefore endosome movement may not need microtubules. We cannot say whether yeast endosomes move along microtubules, but if they do, any advantage of this process is not detected under our conditions.

It is interesting to note that endocytosis in yeast and endocytosis from the apical membrane in animal cells both require actin. The apical surface of animal cells has other properties in common with the yeast cell surface. Both surfaces are in contact with the outside, sometimes hostile environment. Both surfaces have proteins anchored to the membrane by glycosylphosphatidylinositol (Lisanti *et al.* 1990; Nuoffer *et al.*, 1991). Targeting of membrane proteins to both surfaces seems to be signal-mediated (Wandinger-Ness *et al.*, 1990; Roberts *et al.*, 1992). This similarity has perhaps some interesting evolutionary implications. The first eucaryotic cells were undoubtedly single cells with their entire cell surface exposed to the external milieu. Therefore, they only had a surface similar to the yeast cell surface or the apical membrane of animal cells. The endocytic mechanism of these cells may have resembled that in yeast, involving clathrin and actin. Actin may have been necessary as a driving force for endocytosis when cells were under high turgor conditions such as those that exist for yeast and plants. As higher organisms developed and created closed protected environments, the endocytic mechanism on the basolateral surfaces may have lost its requirement for actin.

Materials and methods

Materials and media

Yeast media have been described elsewhere (Maniatis *et al.*, 1982; Dulic *et al.*, 1991). ^{35}S-labelled α-factor was prepared from biosynthetically labelled yeast cells overproducing the pheromone (Dulic *et al.*, 1991).

Table I. Yeast strains used in this work

Strain	Genotype	Source
RH144-3D	*MATa his4 ura3 leu2 bar1-1*	Laboratory collection
RH448	*Mata his4 ura3 leu2 lys2 bar1-1*	Laboratory collection
RH449	*Matα his4 ura3 leu2 lys2 bar1-1*	Laboratory collection
DBY1989	*Matα lys2-802 act1-1*	D.Botstein
DBY1991	*Matα his4-619 act1-2 MOD$^+$*	D.Botstein
EK14-5D	*Mata his4 ura3 leu2 lys2 act1-1 bar1-1*	This work
EK15-2B	*Mata his4 ura3 leu2 lys2 act1-2 bar1-1*	This work
RH949	*MATa his4 ura3-52 tub2-104*	D.Botstein
RH952	*MATa his4-539 ura3-52 lys2-801 tub2-403*	D.Botstein
EK5-1B	*MATa his4 ura3 leu2 tub2-104 bar1-1*	This work
EK6-2C	*MATa his4 ura3 leu2 lys2 tub2-403 bar1-1*	This work
RH1536	*MATa his4 ura3 leu2 lys2 sec10-2 bar1-1*	Laboratory collection
DDY216	*Matα his3 ura3 leu2 lys2 trp1 Δsac6::URA3*	D.Drubin
EK30-1C	*MATa ura3 leu2 trp1 bar1-1 Δsac6::URA3*	This work
DDY75	*MATa his4-619 ura3-52 lys2-801 Δabp1::URA3*	D.Drubin
DDY79	*MATa his4-619 ura3-52 lys2-801*	D.Drubin
RH2108	*MATa his4-619 ura3-52 lys2-801 Δabp1::URA3 bar1::LYS2*	This work
RH2109	*MATa his4-619 ura3-52 lys2-801 bar1::LYS2*	This work

Yeast strains and plasmids

Yeast strains used are listed in Table I. The actin mutants (DBY1989 and DBY1991), β-tubulin mutants (RH949 and RH952) and secretion mutant (RH246-8B) were crossed into a *bar1* background (RH448 or RH449, depending on mating type) by at least two successive crosses and several mutant segregants were tested for their uptake phenotypes. The *sac6* deletion strain (RH2110) was backcrossed once to RH144-3D (isogenic with RH448 except at *LYS2*) and several mutant segregants were tested for their uptake phenotypes. No differences in uptake of α-factor among similar segregants were detected. The *abp1* deletion strain (DDY75) and the isogenic wild-type strain (DDY79) were transformed with a *bar1* disruption construct derived from plasmid pEK3. This pBR322-based plasmid contains the *LYS2* gene, derived from plasmid pDP6 (Fleig *et al.*, 1986) inserted into the unique *SalI* site in the *BAR1* gene, derived from plasmid pZV24 (obtained from V.L.MacKay, Zymogenetics, Seattle, WA). The disruption construct can easily be removed and used for transformation by cutting with *Eco*RI.

LY-CH accumulation

LY accumulation assay was essentially performed as described by Dulic *et al.* (1991). The actin mutants were grown at 24°C in YPUAD medium to 5×10^6 cells/ml, centrifuged and 1×10^8 cells/ml were incubated at 24 or 37°C for 1 h in the presence of 4 mg/ml LY. The β-tubulin mutants were grown at 30°C in YPUAD to 5×10^6 cells/ml, centrifuged and resuspended to the same density and preincubated at 14°C for 20 h. 1×10^8 cells/ml were then incubated at 14°C for a further 3 h in the presence of 4 mg/ml LY. The wild-type control cells were treated the same way as the mutant cells. All cells were then washed and applied to poly L-Lysine coated microscopic slides. Cells were observed and photographed using a Zeiss Axiophot and fluorescence or Nomarski optics as described by Dulic *et al.* (1991).

α-factor internalization assay

Internalization of α-factor was assayed as described by Dulic *et al.* (1991) using biosynthetically labelled [^{35}S]α-factor. The temperature-sensitive actin mutant cells and *sac6* deletion cells were grown at 24°C to 5×10^6 cells/ml, centrifuged and resuspended to 5×10^8 in ice cold YPUAD medium. [^{35}S]α-factor was added and allowed to bind to the cells for 1 h on ice. Cells were then centrifuged and resuspended to 5×10^8 cells/ml in prewarmed 24 or 37°C (actin mutants) or only 24°C (*sac6* deletion strain) YPUAD medium. Samples were taken and counted after 1, 5, 10, 15, 30 and 60 min. The *abp1* deletion strain was treated the same way except that the cells were grown and assayed at 30°C. The *sec10* mutant was grown at 24°C to 5×10^6 cells/ml, centrifuged and preincubated in 37°C prewarmed medium for 20 min. Radioactive α-factor was then added and samples were taken and counted after 5, 10, 15, 30 and 60 min. The β-tubulin mutants were treated as *sec10* except that they were grown at 30°C, preincubation was for 20 h at 11 or 30°C and assays were done at 11 and 30°C, respectively. Samples were taken and counted after 15, 30, 60 and 120 min (for uptake at 11°C) and after 5, 10, 15, 30 and 60 min (for uptake at 30°C).

α-factor degradation assay

The α-factor degradation assay was performed as described by Dulic *et al.* (1991). *Act1-1* and wild-type cells were grown at 24°C in YPUAD medium to 5×10^6 cells/ml, centrifuged and resuspended to 5×10^8 cells/ml. [^{35}S]α-factor was added and allowed to bind for 1 h on ice. The cells were then pelleted at 4°C and resuspended in precooled 15°C YPUAD medium to allow the α-factor to accumulate in the endocytic intermediates (Singer and Riezman, 1990). The cells were centrifuged again at 4°C and resuspended in prewarmed 37°C medium. Samples were taken at 0, 10, 20, 30 and 60 min after shift to 37°C and the α-factor was extracted for thin layer chromatography as described by Dulic *et al.* (1991). Intact and degraded pheromone were resolved using preparative silica gel 60 plates (2.2 mm thick, 20 × 20 cm, Merck, Germany) and the *n*-butanol:propionic acid:water, 50:25:35 (vol/vol/vol) solvent system (Dulic *et al.*, 1991). EN^{3}HANCE spray (DuPont de Nemours International, SA, Regensdorf, Switzerland) was used to coat the plates for fluorography using Kodak XAR-5 film (Eastman Kodak Co.).

Tub2 mutant cells and wild-type control cells were grown at 30°C in YPUAD medium 5×10^6 cells/ml, centrifuged, resuspended in YPUAD medium to 5×10^6 cells/ml and incubated for 20 h at 14°C. Cells were then centrifuged and resuspended to 5×10^8 cells/ml in precooled 14°C medium. [^{35}S]α-factor was added and samples were taken after 20, 60, 120 and 180 min and proceeded as described above.

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References

Adams,A.E.M. and Botstein,D. (1989) *Genetics*, **121**, 675–683.
Adams,A.E.M., Botstein,D. and Drubin,D.G. (1991) *Nature*, **354**, 404–408.
Adams,R.J. and Pollard,T.D. (1989) *Nature*, **340**, 565–568.
Bretscher,A. and Weber,K. (1980) *J. Cell Biol.*, **86**, 335–340.
Brown,S.S. (1993) *Curr. Opin. Cell Biol.*, **5**, 129–134.
Bucci,C., Parton,R.G., Stunnenberg,H., Simons,K., Hoflack,B. and Zerial,M. (1992) *Cell*, **70**, 715–728.
Chvatchko,Y., Howald,I. and Riezman,H. (1986) *Cell*, **46**, 355–364.
Collin,C.A. (1991) *Trends Cell Biol.*, **1**, 57–60.
Cross,F., Roberts,J. and Weintraub,H. (1989) *Annu. Rev. Cell Biol.*, **5**, 341–396.
Drubin,D.G., Miller,K.G. and Botstein,D. (1988) *J. Cell Biol.*, **107**, 2551–2561.
Drubin,D.G., Mulholland,J., Zhu,Z. and Botstein,D. (1990) *Nature*, **343**, 288–290.
Dulic,V., Egerton,M., Elguindi,I., Raths,S., Singer,B. and Riezman,H. (1991) *Methods Enzymol.*, **194**, 697–710.
Fleig,U.N., Pridmore,R.D. and Philippsen,P. (1986) *Gene*, **46**, 237–245.
Goldstein,J.L., Brown,M.S., Anderson,R.G.W., Russell,D.W. and Schneider,W.J. (1985) *Annu. Rev. Cell Biol.*, **1**, 1–41.
Gottlieb,T.A., Ivanov,I.E. Adesnik,M. and Sabatini,D.D. (1993) *J. Cell Biol.*, **120**, 695–712.
Gruenberg,J. and Howell,K.E. (1989) *Annu. Rev. Cell Biol.*, **5**, 453–481.
Huffaker,T.C., Hoyt,M.A. and Botstein,D. (1987) *Annu. Rev. Genet.*, **21**, 259–284.
Huffaker,T.C.,Thomas,J.H. and Botstein,D. (1988) *J. Cell Biol.*, **106**, 1997–2010.
Jenness,D.D. and Spatrick,P. (1986) *Cell*, **46**, 345–353.
Jenness,D.D., Burkholder,A. and Hartwell,L.H. (1983) *Cell*, **35**, 521–529.
Kosaka,T. and Ikeda,K. (1983) *J. Cell Biol.*, **97**, 499–507.
Lisanti,M.P., Le Bivic,A., Saltiel,A.R. and Rodriguez-Boulan,E. (1990) *J. Membr. Biol.*, **113**, 155–167.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marsh,L., Neiman,A.M. and Herskowitz,I. (1991) *Annu. Rev. Cell Biol.*, **7**, 699–728.
Miyajima,I., Nakafuku,M., Nakayama,N., Brenner,C., Miyajima,A., Kaibuchi,K., Arai,K., Kaziro,Y. and Matsumoto,K. (1987) *Cell*, **50**, 1011–1019.
Novick,P. and Botstein,D. (1985) *Cell*, **40**, 405–416.
Novick,P., Field,C. and Schekman,R. (1980) *Cell*, **21**, 205–215.
Nuoffer,C., Jenö,P., Conzelmann,A. and Riezman,H. (1991) *Mol. Cell. Biol.*, **11**, 27- 37.
Payne,G.S. and Schekman,R. (1985) *Science*, **230**, 1009–1014.
Payne,G.S., Baker,D., van Tuinen,E. and Schekman,R. (1988) *J. Cell Biol.*, **106**, 1453–1461.
Pearse,B.M.F. and Robinson,M.S. (1990) *Annu. Rev. Cell Biol.*, **6**, 151–171.
Pierre,P., Scheel,J., Rickard,J.E. and Kreis,T.E. (1992) *Cell*, **70**, 887–900.
Pryer,N.K., Wuestehube,L.J. and Schekman,R. (1992) *Annu. Rev. Biochem.*, **61**, 471–516.
Raths,S., Rohrer,J., Crausaz,F. and Riezman,H. (1993) *J. Cell Biol.*, **120**, 55–65.
Riezman,H. (1985) *Cell*, **40**, 1001–1009.
Roberts,C.J., Nothwehr,S.F. and Stevens,T.H. (1992) *J. Cell Biol.*, **119**, 69–83.
Sandvig,K. and van Deurs,B. (1990) *J. Biol. Chem.*, **265**, 6382–6388.
Sandvig,K., Olsnes,S., Petersen,O.W. and van Deurs,B. (1989) *Methods Cell Biol.*, **32**, 365–382.
Seeger,M. and Payne,G.S. (1992a) *J. Cell. Biol.*, **118**, 531–540.
Seeger,M. and Payne,G.S. (1992b) *EMBO J.*, **11**, 2811–2818.
Shpetner,H.S. and Valle,R.B. (1989) *Cell*, **59**, 421–432.
Singer,B. and Riezman,H. (1990) *J. Cell Biol.*, **110**, 1911–1922.

Wandinger-Ness,A. and Simons,K. (1990) In Hanover,J. and Steer,S. (eds), *Intracellular Trafficking of Proteins*. Cambridge University Press, Cambridge, UK.
Wertman,K.F., Drubin,D.G. and Botstein,D. (1992) *Genetics*, **132**, 337–350.
Whiteway,M., Hougan,L., Dignard,D., Thomas,D.Y., Bell,L. Saari,G.C., Grant,F.S., O'Hara,P. and MacKay,V.L. (1989) *Cell*, **56**, 467–477.
Zanolari,B., Raths,S., Singer-Krüger,B. and Riezman,H. (1992) *Cell*, **71**, 755–763.
Zot,H.G., Doberstein,S.K. and Pollard,T.D. (1992) *J. Cell Biol.*, **116**, 367–376.

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