Molecular Dissection of the Internalization Step of Endocytosis in *Saccharomyces cerevisiae*: Rvs167p and binding partners

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Summary

Several screens performed in *Saccharomyces cerevisiae* have led to the isolation of a large number of endocytic mutants. Interestingly, the majority of these mutants were shown to be defective in the internalization step. In this PhD thesis we have started to dissect the internalization step of endocytosis on a molecular level by looking for protein-protein interactions among the proteins required for this step. We found that Rvs167p, one of the two yeast amphiphysin homologs, is a central player in this interaction network and therefore focused the following studies on Rvs167p and its binding partners.

In a first series of experiments, we demonstrated that Rvs167p and Rvs161p, the two yeast amphiphysin homologs, function together *in vivo*. The interaction of these proteins is relevant and required for their stability.

We next found that Rvs167p interacts with Sla2p/End4p and Myo5p, two proteins involved in the internalization step, and that these three proteins are part of an approximately 600 kDa protein complex most likely involved in regulating the actin cytoskeleton.

In a next series of experiments, we showed that Myo5p, a type I myosin, has a second ATP-independent actin binding site via the SH3-domain in its tail. We show that the interaction with actin requires Vrp1p/End5p and is physiologically relevant.

Furthermore, we have identified the two protein kinases Pkh1p and Pkh2p, the yeast PDK1 homologs, to be part of a sphingoid base-mediated signaling pathway required for the internalization step of endocytosis. We found that Rvs167p is a substrate for Pkh2p *in vitro* and show that mutating the phosphorylation site leads to an endocytic defect *in vivo* suggesting that Rvs167p is one of the downstream effectors of this signaling cascade.

Introduction

The first part of the introduction is a manuscript published in ‘Frontiers in Molecular Biology: Endocytosis’. The second part summarizes recent findings published since the first part was written.

Endocytosis in *Saccharomyces cerevisiae*: involvement of actin, actin-associated protein complexes and lipids in the internalization step

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1. Introduction

All eukaryotic cells are able to internalize extracellular material together with portions of their plasma membrane through a mechanism called endocytosis. The budding yeast *Saccharomyces cerevisiae* (further referred to as yeast) is an organism well suited for the study of cell biological processes like endocytosis. It is an unicellular eukaryote with a membrane organization and organelles similar to higher eukaryotes (see Figure 1), and it offers well developed genetic manipulation techniques that enable the identification of conditional mutants defective in the process of interest. Indeed, several studies have led to the isolation of endocytosis-deficient mutants in yeast based on three major approaches, defective accumulation of fluorescent dyes in their vacuole (Chvatchko *et al*., 1986; Wendland *et al*., 1996), defective pheromone receptor endocytosis (Davis *et al*., 1993; Raths *et al*., 1993) and their synthetic lethality with a mutation in the vacuolar H^+-ATPase (Munn and Riezman, 1994).

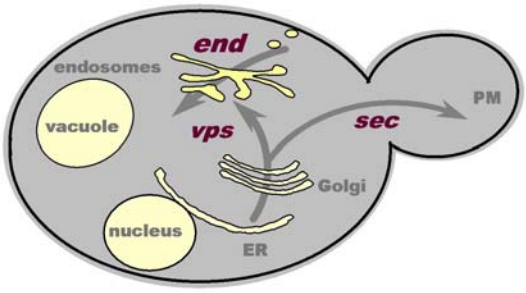

Figure 1. Membrane trafficking in yeast
A schematic overview of a budding yeast cell with internal organelles is drawn showing the major membrane trafficking pathways in yeast. *end*, endocytic mutants; *sec*, secretory mutants; *vps*, vacuolar protein sorting mutants.

Yeast can exist in three different cell types, two haploids with opposite mating types **a** or α and a diploid **a**/α. The haploid **a** or α cells can either grow by mitotic cell division or they can mate with a cell of the opposite mating type thus forming an **a**/α diploid cell. The diploid cell is no longer competent for mating. It also grows by mitotic cell division and upon starvation for nutrients (especially nitrogen) the diploid yeast undergoes meiosis and produces four haploid spores, two of each mating type (see Herskowitz, 1988, and references therein). The differences between **a**, α and **a**/α cells are determined by the mating type locus. Two different alleles encode for different regulatory proteins causing cell-specific expression or repression of a subset of genes ultimately generating the respective cell type (Herskowitz, 1989). In contrast to the diploid cells, both haploid mating types secrete and recognize short peptides called pheromones which bind to receptors on the cell surface of cells of the opposite mating type. Both the α-factor receptor Ste2p and the **a**-factor receptor Ste3p are plasma membrane proteins with seven membrane-spanning segments and are coupled to a heterotrimeric G protein that is involved in the mating-specific signal transduction pathway (Marsh *et al*., 1991). α cells secrete α-factor but recognize **a**-factor which is secreted by **a** cells and vice versa. Binding of either ligand to its receptor leads to several striking changes, including transcriptional induction of a variety of genes required for mating, arrest in the G1 phase of the cell cycle, clearance of pheromone binding sites, and formation of cell surface projections which are the sites of cell-cell fusion (Kurjan, 1992; Marsh *et al*., 1991). Cells displaying these morphological changes have been termed ‘shmoos’. Thus the mating pheromone system function in cell to cell communication to synchronize the cell cycles of the mating partners and to allow the appropriate fusion events.

Receptor-mediated endocytosis was shown to occur in yeast by using radioactively labeled α-factor pheromone (Chvatchko *et al*., 1986; Jenness and Spatrick, 1986). The

study of the pheromone receptors, especially the α-factor receptor Ste2p, has shed light on the sequence of events resulting in receptor down-regulation. It was shown that Mat **a** cells are competent to respond to α-factor and internalize it throughout the cell cycle (Zanolari and Riezman, 1991). The pheromone receptors undergo constitutive endocytosis at a slow rate, and upon ligand binding the internalization rate is greatly stimulated and hyperphosphorylation and ubiquitination of the cytoplasmic tail of the receptors are induced (for review see Riezman, 1998). Multiple internalization signals have been identified in the cytoplasmic tail of Ste2p. The most membrane proximal signal, SINNDAKSS, was shown to be necessary and sufficient for internalization of a truncated receptor. The crucial residue is the lysine that when mutated to arginine completely blocks receptor internalization (Rohrer *et al*., 1993). The lysine was shown to be an acceptor site for ubiquitination and this ubiquitination signals the internalization of the receptor (Hicke and Riezman, 1996). Several other yeast and mammalian plasma membrane proteins have been shown to be ubiquitinated and internalized suggesting a general role of ubiquitination not only in yeast but also in higher eukaryotes (for review see Hicke, 1997, and Hicke, 1999). Another internalization signal in yeast, NPFXD, was found in Kex2p and a similar NPF-sequence was shown to be necessary for the pheromone dependent internalization of a truncated **a**-factor receptor (Tan *et al*., 1996).

The endocytic pathway in mammalian cells has been well characterized at the morphological level (see Gruenberg and Maxfield, 1995; Mellman, 1996, and references therein) while, until very recently, the characterization of this pathway in yeast was quite poor. Biochemical evidence for two endocytic intermediates between the plasma membrane and the vacuole in yeast came from studies following the internalization of radioactively labeled α-factor. Internalized α-factor travels successively and transiently through two biochemically distinct membrane-bound compartments to the vacuole (Singer-Krüger *et al*., 1993). Based on the kinetics of α-factor movement through those compartments and by analogy to the mammalian pathway, the two intermediates were termed early endosomes and late endosomes. Immunofluorescence studies following the internalization and delivery of the α-factor receptor Ste2p have revealed a peripheral early endocytic compartment and a late endocytic compartment near the vacuole. The formation of the early endocytic compartment was dependent upon *SEC18* gene function (yeast homolog of the mammalian N-ethylmaleimide-sensitive fusion protein) which is essential for multiple vesicular fusion events (see Hicke *et al*., 1997, and references therein). Recently, an electron microscopy study has allowed the yeast cell endocytic pathway to be seen at an ultrastructural level by following the internalization and delivery of positively charged Nanogold™ (Nanoprobes, Stony Brook, NY, USA) to the vacuole (Prescianotto-Baschong and Riezman, 1998). In agreement with the previous reports, the first endocytic intermediates seen are small vesicles of approximately 50 nm in diameter which also accumulate at non-permissive temperature in the *sec18* mutant. These vesicles are likely to be the primary endocytic vesicles. In wild-type cells, the Nanogold™ is next found in a peripheral compartment with a tubular-vesicular structure. Later the Nanogold™ is found in a large oval structure with internal membranes located near the vacuole and finally in the vacuole. Taken together, these data suggest a very similar organization of the endocytic pathways in mammalian cells and yeast. In yeast, the first detected intermediates are endocytic vesicles that either generate an early peripheral endocytic intermediate by

homotypic fusion or fuse with a pre-existing endocytic compartment. These tubular-vesicular structures at the cell periphery were termed early endosomes. The next clearly defined intermediate is a large oval structure with internal membranes located near the vacuole, called late endosome. Finally, the endocytosed material is delivered to the vacuole.

It has been previously shown that vacuolar and endocytosed proteins accumulate in an aberrant compartment in a subset of vacuolar protein sorting (*vps*) mutants (Piper *et al*., 1995). This compartment, termed 'class E compartment', has been proposed to be an exaggerated form of a prevacuolar compartment where the endocytic and vacuole biogenesis pathways intersect (Piper *et al*., 1995). The prevacuolar compartment could be an intermediate between the early and late endosomes as defined above. Transport between the endosomal compartments and the vacuole has been shown to require two small GTP-binding proteins of the Rab/Ypt family. Ypt51p functions in the early to late endosome transport step while Ypt7p is involved in late endosome to vacuole trafficking and in homotypic vacuolar fusion (Schimmöller and Riezman, 1993; Singer-Krüger *et al*., 1994; Singer-Krüger *et al*., 1995; Wichmann *et al*., 1992). Several t-SNARES are involved in the endocytic pathway. Vam3p has been localized to the vacuole and shown to be important for several trafficking pathways leading to the vacuole (Nichols *et al*., 1997; Wada *et al*., 1997), while Pep12p has been localized to a prevacuolar compartment and functions in the traffic from the Golgi to this prevacuolar compartment (Becherer *et al*., 1996). Recently, two other members of the yeast syntaxin family of t-SNARES, Tlg1p and Tlg2p, have been identified. Both have been implicated in the TGN/endosomal system but there is controversy as to their localization in the cell and on their exact function (Abeliovich *et al*., 1998; Coe *et al*., 1999; Holthuis *et al*., 1998a; Holthuis *et al*., 1998b; Seron *et al*., 1998). Nevertheless, not only the morphology of the endocytic pathway but also the components involved in regulating and mediating the trafficking steps appear to be conserved from yeast to mammals.

In this review, we will focus on the internalization step of receptor-mediated endocytosis. Different studies have led to the identification of a great number of mutants impaired in this step (see Table 1). Analysis of these mutants revealed two fundamental aspects of the internalization process: The requirement for actin and a subset of actin-associated proteins, and the importance of certain lipids. Both aspects will be discussed together with the role of clathrin and an overview of the techniques available to study endocytosis in yeast.

Table 1. Yeast genes required for the uptake step of receptor-mediated endocytosis

Yeast Gene	Homologies/Comments	Motifs/Domains
ACT1/*END7*	Actin	
AKR1		Ankyrin repeat
ARC35/*END9*	Subunit ARP2/3-complex	
ARP2	Actin related protein	
ARP3	Actin related protein	
CHC1	Clathrin heavy chain	
CLC1	Clathrin light chain	
CMD1	Calmodulin	Four EF hands
END3	Eps15	EH domain
ERG2/*END11*	Ergosterol biosynthesis enzyme	
LAS17/ *BEE1*	Human WASP	
LCB1/*END8*	Ceramide biosynthesis enzyme	
MYO5	Type I myosin	SH3
PAN1/*DIM2*	Eps15	EH domains
RSP5/*NPI1*	Ubiquitin protein ligase	HECT domain
RVS161/*END6*	Amphiphysin	
RVS167	Amphiphysin	SH3
SAC6	Fimbrin	
SJL1, *SJL2*, *SJL3*	Synaptojanin	
SLA2/END4	Talin	
SRV2/*END14*		
VRP1/END5		Proline rich

2. Techniques used to study endocytosis in yeast

Several reporter systems have been developed to study endocytosis in yeast. Fluid phase endocytosis can be followed by using fluorescent dyes like lucifer yellow (Riezman, 1985) or FM 4-64 (Vida and Emr, 1995), or by internalization of electron dense particles and analysis by electron microscopy (Prescianotto-Baschong and Riezman, 1998; Wendland *et al*., 1996). Receptor-mediated endocytosis can be assayed by following **a**-factor receptor Ste3p (Davis *et al*., 1993) and α-factor pheromone (Chvatchko *et al*., 1986) or by measuring the clearance of transporters from the plasma membrane (Berkower *et al*., 1994; Lai *et al*., 1995; Riballo *et al*., 1995; Volland *et al*., 1994). In the present chapter we will present and detail these different methods to follow endocytosis in yeast.

2.1. Fluid-phase endocytosis

2.1.1. Fluorescent dye uptake

Two fluorescent dyes are commonly used to study fluid-phase endocytosis in yeast, lucifer yellow-carbohydrazide (LY), a fluid-phase marker, and FM 4-64, a membrane probe.

LY is a small hydrophilic fluorescent molecule that is incapable of diffusion across biological membranes. It is non-toxic, highly fluorescent and resistant to bleaching. Uptake of LY is time-, energy-, and temperature-dependent and it is nonsaturable. The rate of endocytic accumulation has been estimated as 27 nl/mg of cellular protein/hour at 30°C for yeast cells (Riezman, 1985), compared to 250 nl/mg/h in murine peritoneal macrophages (Swanson *et al*., 1985). Internalized LY accumulates in the vacuole and can be visualized by fluorescence

microscopy using FITC optics. The vacuole in each cell is visible as an indentation when observed with Nomarski optics. LY has been used to screen for mutants that are defective in endocytosis, since the assay is simple to perform (Chvatchko *et al*., 1986). Unfortunately, due to the limitations in the resolution of yeast organelles by light microscopy, it has not been possible to visualize internalized LY in any intermediate compartment.

Recently, the lipophilic styryl dye FM 4-64 (*N*- (3-thiethylammoniumpropyl) -4-(*p*-diethyl-aminophenylhexatrienyl) pyridinium dibromide) has been shown to enter yeast cells by an endocytic mechanism (Vida and Emr, 1995). This dye selectively labels the membrane of the intracellular organelles along the endocytic pathway since it is fluorescent only when inserted into membranes. During a time-course of FM 4-64 staining, the dye initially stains the yeast plasma membrane, then the cytoplasmic intermediate endosomal compartments and finally the vacuolar membrane (Vida and Emr, 1995). FM 4-64 has the advantage that it can be used to visualize intermediates between the plasma membrane and the vacuole during endocytosis.

2.1.2. Electron dense endocytic markers

Positively charged Nanogold™ (Nanoprobes, Stony Brook, NY) is a new marker used to follow the endocytic pathway in yeast (Prescianotto-Baschong and Riezman, 1998). Nanogold™ binds to the plasma membrane of yeast spheroplasts and its internalization and intracellular targeting can be followed by electron microscopy. Nanogold™ cannot be degraded, and can be used to visualize all compartments along the endocytic pathway.

Cationized ferritin was also used to follow endocytosis in yeast spheroplasts (Wendland *et al*., 1996). This marker is also electron dense and can be visualized by electron microscopy. Cationized ferritin has been used to identify structures that accumulate in endocytic mutants.

2.2. Receptor-mediated endocytosis

2.2.1. α-factor pheromone uptake

Receptor-mediated endocytosis can be followed using the yeast pheromone α-factor. α-factor binds to its specific cell surface receptor, the *STE2* gene product (Jenness *et al*., 1983). Internalized α-factor is transported through two intermediate compartments, the early and late endosomes (Hicke *et al*., 1997; Singer-Krüger *et al*., 1993), on its way to the vacuole where it is degraded by resident vacuolar proteases (Schandel and Jenness, 1994; Singer and Riezman, 1990). The best way to quantitatively assess the earliest stages of endocytosis in yeast is to follow the uptake of radioactively labeled α-factor by Mat **a** cells.

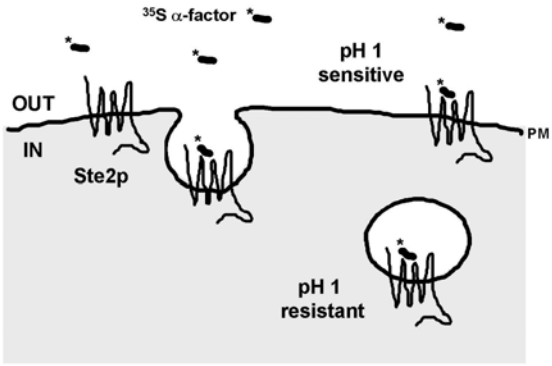

Figure 2. α-factor uptake assay
[^{35}S]α-factor binds the α-factor receptor Ste2p and is internalized. The internalization rate is determined by dividing the fraction of radiolabelled α-factor that is internalized and therefore cannot be removed from the cells by an acid wash protocol (pH 1-resistant), by the total cell-associated counts (pH 1-sensitive).

The α-factor pheromone is radioactively labeled with $^{35}SO_4$ *in vivo*, recovered from the culture supernatant and purified to obtain ^{35}S-labeled α-factor (Dulic *et al*., 1991). [^{35}S]α-factor uptake assays are performed on mid-log phase cells using either the continuous presence or the pulse-chase protocol (Dulic *et al*., 1991). The percentage of internalized α-factor at each time point is calculated by dividing the internalized counts (pH 1-resistant) by the total cell-associated counts (pH 6-resistant) (see Figure 2).

*2.2.2. **a**-factor receptor internalization*

Receptor-mediated endocytosis can also be assayed by following the yeast **a**-factor receptor, Ste3p. The **a**-factor receptor, like the Ste2p, is subjected to two modes of endocytosis, a constitutive, ligand-independent mechanism and a regulated, ligand-dependent mechanism (Davis *et al*., 1993). Both mechanisms result in delivery of the receptor to the vacuole for subsequent degradation. The endocytic assay was developed based on the down-regulation of the receptor by determining the rate of degradation of Ste3p. The ligand-independent turnover of the Ste3p receptor is rapid, with a $t_{1/2}$ estimated to be ~15 min. An endocytic defect leads to a turnover defect of Ste3p and to accumulation of the receptor at the cell surface. Ste3p is labeled with [^{35}S]methionine in a pulse-chase protocol, at various time points after the initiation of the non-radioactive chase, aliquots of the labeled intact cells are taken and subjected to external protease treatment. This assay distinguishes surface-localized receptors (susceptible to external proteases), from receptors that localize to compartments inside the cell (resistant to external proteases). The Ste3p protein is immunoprecipitated from cell extracts and subjected to polyacrylamide gel electrophoresis (Davis *et al*., 1993).

2.2.3 Permeases and transporters uptake

Another rapid and sensitive way to follow endocytosis is to measure clearance of permeases and transporters from the plasma membrane. A number of stimuli appear to trigger rapid endocytosis and subsequent vacuolar degradation of various permeases when the uptake of their respective substrate is no longer needed by the cell. Such a control of permease stability in response to nutrients has been demonstrated for inositol, tryptophane and uracil permeases (Beck *et al*., 1999; Lai *et al*., 1995; Seron *et al*., 1999; Volland *et al*., 1994). Similar mechanisms were also reported for the Ste6p **a**-factor pheromone transporter, the Pdr5p ATP-binding cassette (ABC) transporter and the maltose transporter (Berkower *et al*., 1994; Egner *et al*., 1995; Kolling and Hollenberg, 1994; Riballo *et al*., 1995).

The best studied among the yeast permeases is the uracil permease, encoded by the *FUR4* gene (Weber *et al*., 1986). Uracil permease is phosphorylated and ubiquitinated at the plasma membrane and undergoes rapid internalization followed by vacuolar degradation in cells submitted to various stress conditions, such as inhibition of protein synthesis (Galan *et al*., 1996; Volland *et al*., 1992). The fate of plasma membrane uracil permease can be followed in exponentially growing cells after inhibition of protein synthesis by cycloheximide. [^{14}C]Uracil uptake is measured at various time points after addition of cycloheximide, and protein extracts are prepared and analyzed for uracil permease on immunoblots. In wild-type yeast cells, inhibition of protein synthesis triggers rapid loss of uracil uptake, concurrent with permease degradation, whereas in endocytic mutants a protection against cycloheximide-induced loss of uracil uptake is observed (Volland *et al*., 1994).

The yeast maltose transporter is degraded in the vacuole after internalization by endocytosis (Riballo *et al*., 1995). This internalization occurs under certain physiological conditions such as impaired protein synthesis or presence of a fermentable substrate in the medium. Endocytosis of this protein is dependent on the actin network but independent of microtubules (Penalver *et al*., 1997). In addition, the binding of ubiquitin is required for the internalization step (Lucero and Lagunas, 1997). By using yeast mutants defective in the heavy chain of clathrin and in several subunits of the COPI and the COPII complexes, it has recently been shown that clathrin and the two cytosolic subunits of COPII, Sec23p and Sec24p, are involved in endocytosis of the maltose transporter (Penalver *et al*., 1999).

3. Actin and actin-associated proteins

3.1. The actin cytoskeleton

Initial studies in yeast revealed a fundamental role for the actin cytoskeleton in the internalization step of endocytosis (Kübler and Riezman, 1993). Using two conditional mutations in both actin and β-tubulin, a requirement for actin in the internalization step but not for post-internalization trafficking was demonstrated. Microtubules were not required at all. An *act1-1* strain showed a rapid onset of the endocytic defect at 37°C, even without pre-incubation, and internalized α-factor at less than 10% of the rate detected in wild-type cells suggesting a direct role of actin in the internalization step.

The actin cytoskeleton in yeast consists of cortical patches and actin cables both composed of F-actin. Patches show a polarized distribution that changes during the cell cycle and the cables generally run along the mother-bud axis (see Amberg, 1998, and references therein). At an ultrastuctural level, the actin patches consist of a finger-like invagination of plasma membrane around which actin filaments and actin binding proteins like Abp1p and cofilin are organized (Mulholland *et al*., 1994). These data led to the notion that actin patches might be the sites of endocytosis. However, a recent study following Ste2p internalization by immuno electron microscopy provided evidence that the cortical actin patches are not the sites of receptor internalization (Mulholland *et al*., 1999). The authors showed that Ste2p is not randomly distributed over the plasma membrane but is concentrated in furrow-like invaginations. This localization of Ste2p agrees with previous immunofluorescence studies showing a spotty distribution of Ste2p on the cell surface (Hicke *et al*., 1997).

Using the drug latrunculin A, the actin filaments in yeast were shown to undergo rapid cycles of assembly and disassembly *in vivo* (Ayscough *et al*., 1997) suggesting a very dynamic actin cytoskeleton even in this nonmotile organism. Using conditional mutations in the yeast cofilin gene *COF1*, rapid turnover of cortical actin structures was shown to be essential for endocytosis (Lappalainen and Drubin, 1997). Cells lacking the yeast homolog of the actin filament bundling protein fimbrin, Sac6p, are also defective for internalization (Kübler and Riezman, 1993). Taken together, these data strongly support the central role of an organized dynamic actin cytoskeleton in the internalization step of endocytosis in yeast. Further support comes from the analysis of isolated yeast mutants defective for endocytosis. One mutant allele isolated, *end7-1*, has been shown to be allelic to *ACT1* (Munn *et al*., 1995) and several endocytic mutants also exhibit defects in the actin cytoskeleton (Riezman *et al*., 1996). However, it is important to point out that not all mutants

with defects in the actin cytoskeleton are affected in endocytosis. Mutations in *myo2*, *pfy1* and *tpm1* cause actin cytoskeleton defects similar to those observed in several endocytic mutants but they endocytose as well as wild-type yeast cells (Munn *et al*., 1995). The involvement of the actin cytoskeleton in mammalian cells has been a matter of discussion for a long time (see Geli and Riezman, 1998, and references therein). Ambiguous results have been obtained depending on the cell line and the actin depolymerizing agent used. A recent study using latrunculin A provided evidence that the actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells (Lamaze *et al*., 1997), though other studies have suggested it is not essential (Fujimoto *et al*., 2000).

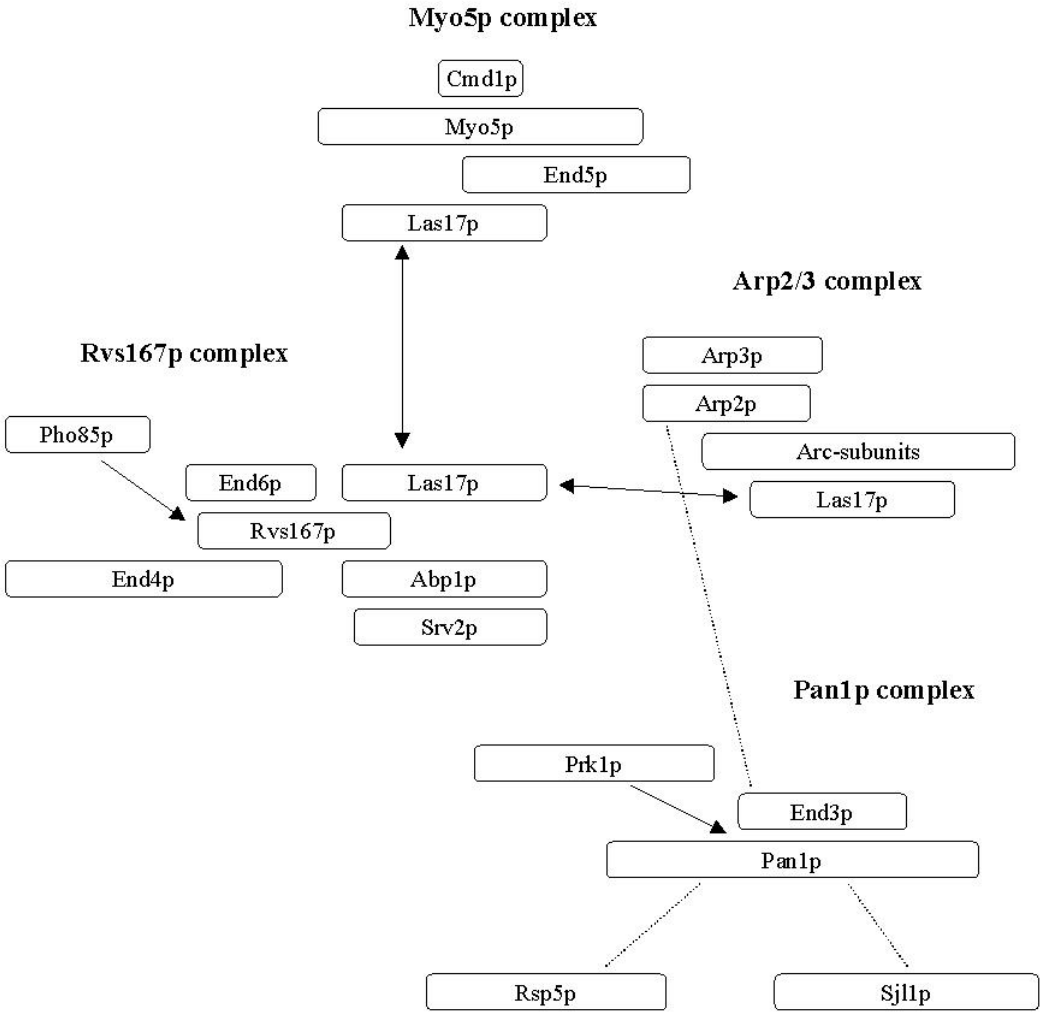

Figure 3. Actin-associated protein complexes
Schematic drawing of the four protein complexes described in detail in the text. Proteins that have been shown to interact biochemically and/or in the two-hybrid system are drawn near each other. Synthetic lethal interactions are shown by a dashed line, and an arrow marks phosphorylation of a protein by a kinase. Las17p might be part of three complexes and therefore connections are drawn by double-headded arrows.

3.2. Actin-associated proteins

As previously mentioned, several endocytic mutants exhibit defects in the actin cytoskeleton and some of the proteins encoded by these genes have been shown to bind to actin or are associated with actin-binding proteins. Several protein complexes have been implicated in the internalization step of endocytosis and will be described below (see Figure 3).

3.2.1. Myo5p complex

Type I myosins have been implicated in actin-dependent membrane motility processes such as membrane trafficking, organelle movement, phagocytosis, pinocytosis and cellular locomotion (see Mermall *et al*., 1998, and references therein). The yeast genome encodes two type I myosins, *MYO3* and *MYO5* (Geli and Riezman, 1996; Goodson *et al*., 1996). Single deletions do not lead to any growth phenotype but deletion of both genes results in a severe growth defect or lethality (see Geli and Riezman, 1996, and references therein). In contrast to a *myo3Δ* strain, a *myo5Δ* strain is impaired for α-factor internalization at 37°C, suggesting a more direct role of Myo5p in the internalization step (Geli and Riezman, 1996). Myo5p contains two IQ motifs that constitute binding sites for the small EF-hand containing protein calmodulin (Cmd1p) and this interaction is required for endocytosis *in vivo* (Geli *et al*., 1998). A previous study had already implicated Cmd1p in the internalization step of endocytosis and this function of Cmd1p appears to be Ca^{2+}-independent because a calmodulin allele defective in high-affinity calcium binding (*cmd1-3*) shows no endocytic defect (Kübler *et al*., 1994). In addition, evidence suggests that at least two distinct calmodulin functions are required for the internalization step. One target of Cmd1p is Myo5p and this function is impaired in the *cmd1-247* mutant. Another function is impaired in the *cmd1-228* mutant for which the target is not known (Geli *et al*., 1998). The actual function of Myo5p in the internalization step is unknown. Since none of the dynamin-homologs in yeast have a role in the internalization step of endocytosis, Myo5p was suggested to replace dynamin in yeast endocytosis if one assumes that dynamin works as a mechanochemical enzyme (Geli and Riezman, 1998). Myo5p has been shown to interact via its SH3-domain with End5p/Vrp1p and this interaction is required for polarized localization of Myo5p (Anderson *et al*., 1998). Interestingly, a mutant allele of *END5*, *end5-1*, has been isolated in a previous screen for endocytic mutants (Munn *et al*., 1995). End5p is very rich in proline residues and contains several putative SH3-binding sites. End5p interacts with actin in the two-hybrid system and an actin-binding domain was mapped to the first 70 amino acids of End5p (Vaduva *et al*., 1997).

In summary, the endocytic function of Myo5p is regulated at least partially by Cmd1p and there is a second function of Cmd1p in the internalization step whose target is unknown. Polarized localization of Myo5p depends on End5p and therefore this interaction might concentrate or activate Myo5p at the sites of function.

3.2.2. Arp2/3 complex

As already mentioned, a dynamic actin cytoskeleton is required for the internalization step of endocytosis. Recently, a protein complex, the Arp2/3 complex, has been implicated in the regulation of the actin cytoskeleton and identified in several organisms. The Arp2/3 complex consists of seven subunits and has been shown to stimulate actin filament nucleation and to bind both pointed-ends and sides of actin filaments (see Machesky and Gould, 1999, and references therein).

Interestingly, several subunits of the complex are required for the internalization step of endocytosis. Mutations in genes encoding the actin-related proteins *ARP2* (Moreau *et al*., 1997) and *ARP3* (C. Schaerer-Brodbeck and H. Riezman, unpublished data) block receptor-mediated endocytosis. A conditional mutant, *end9-1*, has been isolated in a screen for endocytic mutants (Munn and Riezman, 1994) and subsequently shown to be allelic to *ARC35*, the 35 kD subunit of the Arp2/3 complex (Schaerer-Brodbeck and Riezman, 2000b). The *end9-1* strain is defective for both fluid phase and receptor-mediated endocytosis at the restrictive temperature (Munn and Riezman, 1994). Taken together these data strongly implicate the Arp2/3 complex in the internalization step. To our knowledge no data have been published concerning the endocytic phenotypes of mutations in the other subunits of the complex.

Studies from two different laboratories have implicated the yeast homolog of the human Wiskott-Aldrich Syndrome protein (WASP), *LAS17/BEE1*, in Arp2/3 complex function (Madania *et al*., 1999; Winter *et al*., 1999). Las17p has been shown to interact with the Arp2/3 complex by co-immunoprecipitation. *LAS17* is an allele-specific multicopy suppressor of *ARP2* and *ARP3* mutations, and overexpression restores the endocytic defect of the *arp2-2* mutant allele. Furthermore, *las17Δ* is synthetically lethal with several *ARP2* mutant alleles and with *arp3-14*. In addition, Las17p stimulated the actin nucleation activity of the Arp2/3 complex *in vitro*. Taken together, these data support an important functional interaction of Las17p and the Arp2/3 complex. Las17p interacts with the Arp2/3 complex via its carboxy terminal WA-domain. Unexpectedly, deletion of this domain, as opposed to a *las17Δ*, caused relatively minor defects suggesting that Las17p does not function solely via the Arp2/3 complex and that other cellular factors act redundantly with Las17p to activate the Arp2/3 complex (Winter *et al*., 1999). Interestingly, a *las17Δ* strain exhibits a strong α-factor internalization defect indicating a role in endocytosis (Madania *et al*., 1999).

LAS17 has been isolated as a multicopy suppressor of the *end5-1* temperature sensitive growth defect and overexpression of Las17p also restores the endocytic defect of this mutant allele. Furthermore, *las17Δ* is synthetically lethal with *end5Δ* and the two proteins interact in the two-hybrid system (Naqvi *et al*., 1998). These findings suggest a functional relationship between Las17p and End5p. A similar interaction has been detected in human cells between WASP and WIP (WASP interacting protein), whose yeast homolog probably is *END5* (Ramesh *et al*., 1997).

In summary, the data presented in the previous section (Myo5p complex) together with this data suggest that End5p can associate with both Myo5p and Las17p and thus might functionally couple the Arp2/3 complex to the Myo5p complex. However, there is no evidence that End5p interacts with both proteins at the same time leaving the possibility that End5p exerts its function in two separate complexes.

3.2.3. Rvs167p complex

The *end6-1* mutant was isolated in a screen for endocytic mutants and shown to be allelic to *RVS161* (Munn *et al*., 1995). Rvs161p shows homologies to a second yeast protein, Rvs167p, and to the mammalian protein amphiphysin. In mammalian cells, amphiphysin interacts with dynamin, synaptojanin, AP-2 and clathrin, all proteins implicated in clathrin-mediated endocytosis, and has been suggested to act as a scaffold protein (see Wigge and McMahon, 1998, and references therein). The entire Rvs161p is homologous to the N-terminal part of

Rvs167p and amphiphysin but lacks the SH3-domain present in Rvs167p and amphiphysin. Mutations in either *RVS161* or *RVS167* lead to similar phenotypes except for a cell fusion defect detected in *rvs161Δ* and not in *rvs167Δ* strains (see Navarro *et al*., 1997, and references therein; Brizzio *et al*., 1998). Both proteins are required for the internalization step of endocytosis (Munn *et al*., 1995) and they interact with each other (Navarro *et al*., 1997) further supporting a joint function. Surprisingly, their localization in the cell is different. Rvs161p was shown to be mainly cytosolic in unbudded cells and upon appearance of the bud to localize mainly to the mother-bud-neck (Brizzio *et al*., 1998). In contrast, in unbudded cells Rvs167p is localized mainly in small cortical patches throughout the cell which polarize at the bud emergence site and in the small buds (Balguerie *et al*., 1999). Taken together, these data support both overlapping and unique functions of the proteins in the cell.

Rvs167p interacts with the Pho85 cyclin-dependent kinase complexes and is a substrate for this kinase *in vitro*. The similarities of the phenotypes associated with the deletion of *PHO85* and *RVS167* as well as the reduced phosphorylation of Rvs167p in a *pho85Δ* strain *in vivo* suggest a regulatory function of Pho85 kinases on Rvs167p activity (Lee *et al*., 1998). The SH3-domain of Rvs167p interacts with Act1p (Amberg *et al*., 1995) and Las17p (Colwill *et al*., 1999; Madania *et al*., 1999) in the two-hybrid system and with Abp1p (Lila and Drubin, 1997) in a binding assay. Another protein interacting with Rvs167p is End4p/Sla2p (Wesp *et al*., 1997), a protein isolated both in a screen for endocytic mutants (Raths *et al*., 1993) and in a synthetic lethal screen with *abp1Δ* (Holtzman *et al*., 1993). The SH3-domain of Abp1p interacts with Srv2p (Freeman *et al*., 1996). Deletion of either *ABP1* or *SRV2* show no endocytic defect (Kübler and Riezman, 1993; Wesp *et al*., 1997) but interestingly, a mutant allele (*srv2-14*) of *SRV2* has been isolated based on its endocytic defect (Wesp *et al*., 1997). Furthermore, deletion of the central coiled-coil domain of End4p creates a synthetic endocytic defect in the absence of Abp1p or Srv2p suggesting a redundant endocytic function of both Abp1p and Srv2p with End4p (Wesp *et al*., 1997). Several lethal double mutant combinations have been shown among disruptions in *ABP1*, *END4*, *RVS167* and *SRV2* (Lila and Drubin, 1997) further supporting a functional relationship of these actin-associated proteins.

As mentioned previously, Rvs167p interacts with Las17p in the two-hybrid system. This interaction could link the Rvs167p complex to the Arp2/3 complex and/or the Myo5p complex via Las17p. However, Las17p probably does not interact with all these complexes at the same time. Rather a dynamic model can be envisioned in which Las17p interactions are tightly regulated and possibly mutually exclusive to allow a controlled interplay between the different complexes.

3.2.4. Pan1p complex

The *end3-1* allele was isolated in a screen for endocytic mutants and shown to be required for the internalization of α-factor and for fluid phase endocytosis (Bénédetti *et al*., 1994; Raths *et al*., 1993). End3p contains an N-terminal EH-domain (Eps15 homology domain) first identified in the mammalian protein Eps15 (Wong *et al*., 1995). Several EH-domain containing proteins are present in yeast, and of these Pan1p has also been shown to be required for endocytosis (Tang *et al*., 1997; Wendland *et al*., 1996). Interestingly, *END3* was identified as a multicopy suppressor of *pan1-4*, and *end3Δ* is synthetically lethal with *pan1-4*. In addition, both proteins have been shown to interact by co-immunoprecipitation and in the two-hybrid system (Tang *et al*., 1997). Loss of function mutations in *PRK1*

suppress the growth and actin defect of *pan1-4* (Zeng and Cai, 1999). Prk1p is a serine/threonine kinase that was shown to phosphorylate Pan1p *in vitro*. This phosphorylation occurs in the Pan1p domain implicated in End3p binding and pre-incubation of this Pan1p-domain with End3p prior to the *in vitro* kinase assay reduced the phosphorylation of the Pan1p-domain (Zeng and Cai, 1999). *PRK1* shows homologies to another putative yeast kinase, *ARK1*. Single deletions of either kinase gene are viable but an *ark1Δ prk1Δ* strain exhibits large cytoplasmic actin clumps and severe defects in cell growth implicating both kinases in regulating the actin cytoskeleton (Cope *et al*., 1999). Interestingly, Ark1p was isolated in a two-hybrid screen using parts of the End4p as bait, and *prk1Δ* is synthetically lethal with *end4Δ* providing a link to the previously described Rvs167p complex. So far, no function of either Prk1p or Ark1p in regulating End4p or the Rvs167p complex has been shown.

Pan1p also interacts with yAP180A and yAP180B, the yeast homologs of the mammalian AP180, via its EH-domain. As with to their mammalian counterparts, the two yeast proteins interact with clathrin and might therefore function as adaptor proteins (Wendland and Emr, 1998). However, this interaction might not be required for endocytosis because the two yAP180 are not required for endocytosis (see Huang *et al*., 1999, and section 4 below).

END3 and *PAN1* show genetic interactions with several proteins involved in endocytosis. *end3-1* is synthetically lethal with *arp2-1*, a component of the previously described Arp2/3 complex (Moreau *et al*., 1997) and *pan1-20* is synthetically lethal with *sjl1Δ* but not with *sjl2Δ* or *sjl3Δ*, the yeast homologs of mammalian synaptojanin (Wendland and Emr, 1998). A previous study has shown that a strain lacking *SJL1* and *SJL2* (*sjl1Δ sjl2Δ*) exhibits a defect in both receptor-mediated and fluid-phase endocytosis, while a *sjl2Δ sjl3Δ* mutant had only a minor defect, and a *sjl1Δ sjl3Δ* had no defect, implicating these proteins in endocytosis like their mammalian counterpart, synaptojanin (Singer-Krüger *et al*., 1998). *PAN1* shows allele-specific synthetic lethality with *RSP5* (Zolladek *et al*., 1997), an ubiquitin-ligase involved in the ubiquitination of several permeases and therefore in their internalization (see Springael *et al*., 1999, and references therein). *RSP5* is an essential gene, and the conserved cysteine in the HECT domain is required for both yeast cell viability and ubiquitination of permeases like Gap1p. Interestingly, deletion of the N-terminal C_2-domain of Rsp5p does not affect viability but it impairs internalization of Gap1p without affecting ubiquitination of the permease indicating a role of Rsp5p in internalization in addition to its function in ubiquitination (Springael *et al*., 1999).

Recently, two yeast homologs of the mammalian epsin, Ent1p and Ent2p have been identified and shown to be required for FM4-64 internalization. Ent1p interacts with clathrin via its final eight amino acids (Wendland *et al*., 1999). A previous study had reported a weak interaction of Ent1p and the EH-domain of Pan1p in the two-hybrid system (Wendland and Emr, 1998) providing a possible link between the yeast epsins and the Pan1p complex.

Taken together, these data suggest that the two protein kinases Ark1p and Prk1p are involved in regulating the actin cytoskeleton and may work, at least partially, via Pan1p. The genetic interactions detected link the yeast homologs of synaptojanin, *SJL1-3*, and the ubiquitin ligase *RSP5* to the Pan1p complex.

4. Involvement of clathrin in endocytosis

In mammalian cells, the most prominent and best studied mechanism of receptor-mediated endocytosis occurs via clathrin-coated pits and vesicles (see Hirst and Robinson, 1998; Schmid, 1997, and references therein). In addition to the clathrin-mediated endocytic pathway, other so called clathrin-independent endocytic pathways are present and function as the major internalization routes for some receptors (see Lamaze and Schmid, 1995, and references therein). Selective inhibition of clathrin-dependent endocytosis causes the up-regulation of these clathrin-independent pathways (Damke *et al*., 1995).

The clathrin molecule is a triskelion formed by three clathrin heavy chain molecules, each associated with a clathrin light chain. The yeast genome contains a single clathrin heavy chain gene (*CHC1*) and also a single clathrin light chain gene (*CLC1*). Strains with a disruption of either *CHC1* or *CLC1,* as well as a strain harboring a *chc1-ts* allele, show a defect in α-factor internalization. However, these strains still internalize radioactive α-factor at about 35-50% of the level detected in wild-type cells (Chu *et al*., 1996; Huang *et al*., 1997; Payne *et al*., 1988; Tan *et al*., 1993). Compared to the strict requirement of actin and several actin-associated proteins for α-factor internalization (see section 3 above) this partial effect of clathrin mutations points to a non-essential role of clathrin in receptor-mediated endocytosis in yeast. Several models have been proposed to explain this partial effect of clathrin mutations:

1) Clathrin is only required to concentrate receptors at the sites of internalization but not for the actual budding of the vesicles.
2) At least two endocytic pathways exist in yeast that are actin-dependent but only one of them is clathrin-dependent.
3) Another protein can partially substitute for mutations in clathrin and take over some of its functions.
4) The defect of clathrin mutations is indirect.

The findings that no other genes with extended homologies to *CHC1* are found in the yeast genome and the rapid onset of the endocytic defect in a *chc1-ts* strain (Tan *et al*., 1993) would argue against the later two models. An interesting recent finding is that the internalization of the uracil permease Fur4p is unaffected at restrictive temperature in a *chc1-ts* strain (A. Gratias and R. Haguenauer-Tsapis, unpublished data). Therefore, the requirement for clathrin in endocytosis seems to be dependent on the protein to be internalized. According to the second model, the two proteins might be internalized by two different endocytic pathways. According to the first model, a cell might need to remove an actively signaling receptor (Ste2p with bound α-factor) from the plasma membrane faster than a pyrimidine base permease (Fur4p) and thus only Ste2p would normally be concentrated for internalization.

In mammalian cells, the heterotetrameric adaptor complexes have been implicated both in recruiting clathrin to membranes and in concentrating receptors in clathrin-coated pits via interactions with their cytoplasmic tails (Hirst and Robinson, 1998). A recent study demonstrated that clathrin can function in the absence of both heterotetrameric adaptors and AP180-related proteins in yeast (Huang *et al*., 1999). A yeast strain with disruptions of all six heterotetrameric AP large chain genes (all β chains, α/γ/δ chains removed) as well as a strain with disruption of the three AP large chain β-subunit genes and the two

YAP180 genes did not display the phenotypes of clathrin-deficient cells. Endocytosis was not affected in these strains and the authors were able to isolate clathrin-coated vesicles from them. Taken together, these data suggest that clathrin can be recruited to membranes in the absence of functional adaptors and that clathrin can function in the absence of adaptors. There is still the possibility that other proteins can function as adaptor molecules and therefore mediate the membrane association of clathrin and the concentration of receptors. In mammalian cells, β-arrestin was shown to act as a clathrin adaptor in the endocytosis of the β2-adrenergic receptor and some other seven transmembrane domain G protein-coupled receptors (Goodman *et al*., 1996). Taken together, all these data clearly suggest that a re-evaluation of the requirements to form a clathrin-coated vesicle must take place.

Additional support for a non-essential role for clathrin in the internalization step in yeast comes from the finding that none of the dynamin-homologs in yeast are involved in endocytosis (see Geli and Riezman, 1998, and references therein). It is well established in mammalian cells that dynamin is required for clathrin-mediated endocytosis (Schmid *et al*., 1998). Nevertheless, several proteins involved in endocytosis in yeast have homologs in mammalian cells and vice versa pointing to at least some functional homologies of endocytosis in both cell types (for a review see Geli and Riezman, 1998).

5. Role of lipids in the endocytic pathway

5.1. Lipid requirement in membrane trafficking in yeast

Recently, not only proteins but also specific lipids were shown to be required for the endocytic pathway. Lipids are responsible for the structural integrity of biological membranes and confer specific dynamic properties to the bilayer. Major lipid components of eukaryotic membranes are phospholipids, sterols, sphingolipids, and glycerolipids. Phosphatidic acid, diacylglycerol (DAG), sphingolipids, and phosphoinositides (PI) have been implicated in several stages of membrane trafficking in yeast.

The vacuolar protein sorting (*VPS*) pathway of yeast mediates transport of vacuolar protein precursors from the late Golgi to the lysosome-like vacuole (see Figure 1). Sorting of some vacuolar proteins occurs via the prevacuolar endosomal compartment and mutations in a subset of *VPS* genes interfere with the Golgi-to-endosome transport step. The *VPS34* gene encodes a PI 3-kinase and this enzyme is required for protein sorting to the vacuole (Schu *et al*., 1993). Inactivation of *VPS34* results in impaired fusion of endosomal transport intermediates with the vacuole . These data implicate PI(3)P as a regulator of membrane traffic (Wurmser and Emr, 1998).

The *SEC14* gene product encodes a phosphatidylinositol/phosphatidylcholine transfer protein that is required for the production of secretory vesicles from the Golgi (Bankaitis *et al*., 1990). This requirement can be relieved by inactivation of the cytosine 5'-diphosphate (CDP)-choline pathway for phosphatidylcholine biosynthesis (Cleves *et al*., 1991) or by increasing the supply of DAG to the Golgi (Kearns *et al*., 1997). Recently, a *sec14*

mutant that was inactivated for phosphatidylinositol (PI), but not phosphatidylcholine (PC) transfer activity, was shown to be able to rescue the lethality and the Golgi secretory defects associated with *sec14-ts* or *sec14Δ* mutations. These findings indicate that PI binding/transfer seems to be dispensable for Sec14p function *in vivo* (Phillips *et al*., 1999).

GPI-anchored proteins are attached to the membrane via a glycosylphosphatidyl-inositol-(GPI) anchor whose carbohydrate core is conserved in all eukaryotes. Apart from membrane attachment, the precise role of the GPI-anchor is not known, but it has been proposed to play a role in protein sorting. *In vitro* and *in vivo* data suggest that ceramides are required for trafficking of GPI-anchored proteins from the endoplasmic reticulum (ER) to the Golgi apparatus in yeast (Horvath *et al*., 1994; Skrzypek *et al*., 1997; Sutterlin *et al*., 1997).

Members of the synaptobrevin/VAMP family of v-SNAREs are involved in vesicle docking and have been shown to be essential for exocytosis in yeast. Recessive mutations in either *ELO2* or *ELO3*, two genes that mediate the elongation of very long chain fatty acids, allow yeast to grow normally and secrete in the absence of v-SNAREs. Thus, the v-SNARE requirement in constitutive exocytosis can be abrogated by mutations in genes involved in lipid synthesis (David *et al*., 1998).

5.2. Lipid requirement in endocytosis

The *VMA2* gene encodes the 60 kD vacuolar H^+-ATPase (V-ATPase) regulatory subunit (subunit B). In cells bearing a disruption of *VMA2* (*vma2Δ*), the V-ATPase does not assemble on the vacuolar membrane and consequently the lumen of the vacuole fails to become acidified. Yeast *vma2Δ* mutants are able to grow if the external medium is around pH 5.5 but not at pH 7 or higher pH (Yamashiro *et al*., 1990), because the cells are able to take up protons from the medium by fluid-phase endocytosis. Based on this result a screen for isolation of endocytosis-deficient (*end*) mutants was developed, taking advantage of the synthetic lethality of endocytic mutants with *vma2Δ*.

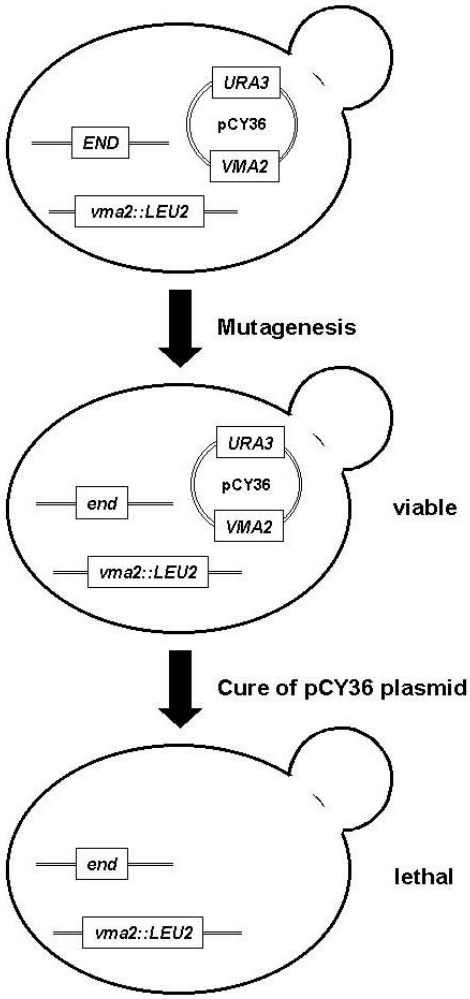

Figure 4. The *vma2* synthetic lethality mutant screen
The *VMA2* chromosomal gene is disrupted by *LEU2* in a yeast strain carrying the wild type *VMA2* gene on a *URA3* low copy plasmid (pCY36). After mutagenesis some of the cells will become endocytosis-deficient (*end*) mutants. These *end* mutants cannot grow without a wild type copy of *VMA2* thus cannot lose the pCY36 plasmid. 5'-fluoro-orotic acid (5'-FOA) is used to screen for *end* mutants, because only the Ura^- cells, which are END^+ can grow on this selective medium.

The principle of this screen is presented in Figure 4 and resulted in isolation of the *end8-1* and *end11-1* mutants among others (Munn and Riezman, 1994). The cloning and sequencing of these two genes revealed that *END8* is allelic to *LCB1*, an enzyme required in sphingolipid synthesis and that *END11* encodes *ERG2* an enzyme involved in ergosterol synthesis (Munn *et al*., 1999). These findings showed that mutants affected in lipid biosynthesis are defective in endocytosis.

5.2.1. Sphingosine bases requirement for endocytosis

The *LCB1* gene is essential and encodes a subunit of the serine palmitoyl-transferase enzyme (SPT; Buede *et al*., 1991). SPT catalyses the first step in *de novo* sphingolipid synthesis, the condensation of serine and palmitoyl-CoA to yield the 3-ketosphinganine (KDS; see Figure 5 and Nagiec *et al*., 1994). In yeast, sphingosine bases (KDS, PHS and DHS) are generated by *de novo* synthesis in the ER.

The *end8-1 = lcb1-100* mutant cells have a temperature-sensitive growth defect. At 24°C this mutant exhibits α-factor uptake kinetics that are almost like wild-type cells, but at 37°C the mutant is clearly defective. Moreover, the *lcb1-100* cells are defective for accumulation of LY in the vacuole at 24°C and 37°C and showed a few small vacuoles when viewed by Nomarski optics. In summary, the *lcb1-100* mutant is defective in the internalization step of both receptor-mediated and fluid-phase endocytosis at non-permissive temperature (Munn and Riezman, 1994).

These findings suggest that sphingosine bases might play an important role in the internalization step of endocytosis. To test this hypothesis, sphingosine bases (PHS and DHS) were added externally to the *lcb1-100* strain and the rate of receptor-mediated endocytosis was determined. Both phytosphingosine (PHS) and dihydrosphingosine (DHS) were able to suppress the endocytic defect observed in *lcb1-100* cells (Zanolari *et al*., 2000). The use of genetic approaches should help to identify the sphingosine base compound (KDS, DHS or PHS) that is required for endocytosis in yeast. The Sur2p activity is required to interconvert DHS and PHS in yeast (see Figure 5; Haak *et al*., 1997). To check whether one of these two compounds is specifically required for the suppression, the double mutant *lcb1 sur2* cells can be tested for α-factor uptake in presence of DHS or PHS. The recent identification of two *Saccharomyces cerevisiae* genes encoding sphingosine kinases, *LCB4* and *LCB5* (Nagiec *et al*., 1998), should indicate whether phosphorylated sphingosine bases are required to trigger endocytosis in yeast.

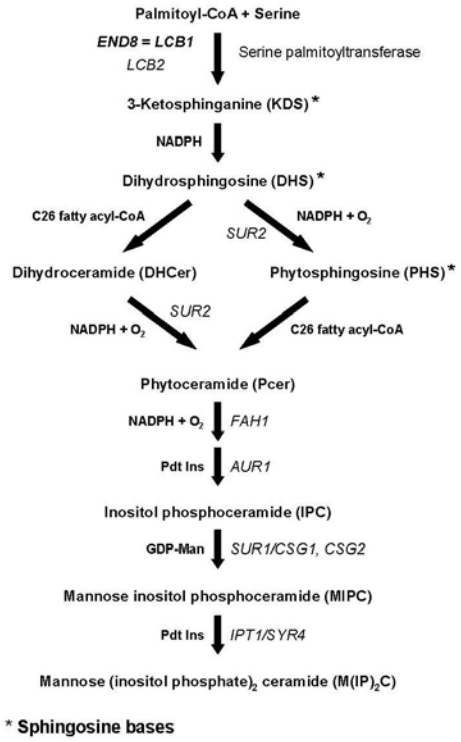

Figure 5. Sphingolipid biosynthetic pathway in yeast
Known pathway intermediates, substrates, and genes implicated in sphingolipid biosynthesis are indicated. Sphingolipids are characterized by the presence of a long chain fatty acid that is amide-linked to a sphingosine long chain base moiety. Sphingosine long chain bases are indicated.

Sphingosine bases and their phosphorylated derivatives (DHS-1P and PHS-1P) are thought to be signaling molecules for regulating a variety of mammalian cellular processes including cell growth, motility and apoptosis (Hannun, 1996; Perry and Hannun, 1998). Sphingolipids and phosphorylated sphingosine bases have also been implicated in the yeast stress response (Jenkins *et al*., 1997; Mandala *et al*., 1998; Skrzypek *et al*., 1999). Sphingosine bases or ceramides have been proposed to activate protein phosphatases. The best candidate for a ceramide-activated protein phosphatase (CAPP) in yeast is the protein phosphatase 2A (PP2A), which has two regulatory subunits, Cdc55p and Tpd3p (Healy *et al*., 1991; van Zyl *et al*., 1992), and a catalytic subunit. The catalytic subunit of CAPP has been postulated to be Sit4p (Nickels and Broach, 1996), but three other genes that are functionally overlapping, *PPH21*, *PPH22* and *PPH3*, encode the major yeast PP2A catalytic activity (Ronne *et al*., 1991; Sneddon *et al*., 1990). In a *lcb1 cdc55Δ* double mutant and in a *lcb1 pph21Δ pph22Δ pph3Δ pph21-ts* strain, endocytosis is restored in the absence of sphingosine synthesis (Friant *et al*., 2000). Therefore, the sphingosine requirement for endocytosis can be suppressed by PP2A mutations. Furthermore, overexpression of two kinases can suppress the sphingosine requirement for both receptor-mediated and fluid-phase endocytosis (Friant *et al*., 2000).

In summary, the sphingosine requirement for endocytosis can be suppressed by loss of PP2A activity or by overexpression of two kinases, suggesting a signaling function of sphingosine in activation of a protein kinase and a protein phosphatase acting sequentially in endocytosis.

5.2.2. Specific sterol requirement for endocytosis

The *end11-1* mutant is defective for the internalization step of endocytosis and has been shown to be allelic to *ERG2* (Munn *et al*., 1999). The *ERG2* gene encodes the sterol C-8 isomerase, an enzyme required for one of the late steps in ergosterol synthesis (see Figure 6; Arthington *et al*., 1991). Ergosterol is the principal sterol in yeast and is an essential lipid component for proper function of the membranes (Daum *et al*., 1998). The *end11(erg2)-1* mutant is defective for LY accumulation in the vacuole and for α-factor uptake at both 24°C and 37°C (Munn and Riezman, 1994). These results suggest an important role of ergosterol in the internalization step of endocytosis.

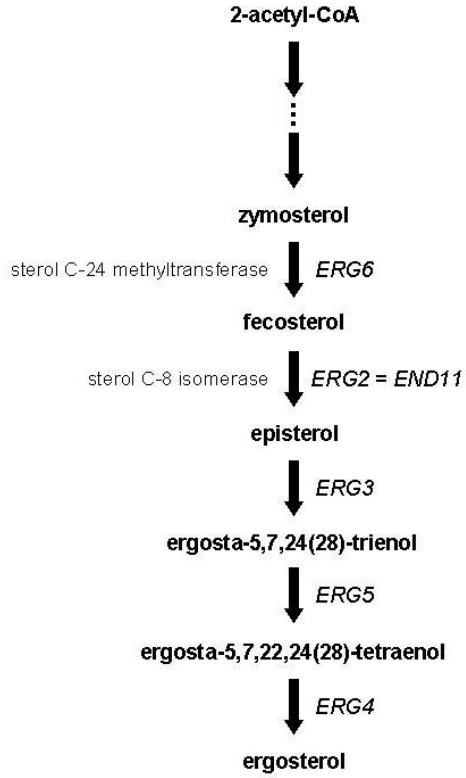

Figure 6. Ergosterol biosynthesis in yeast
The ergosterol biosynthesis pathway is described from zymosterol to ergosterol. The first steps of this pathway to the mevalonate pathway and the farnesyl pyrophosphate to zymosterol pathway are not described. The last steps of ergosterol synthesis represented here are non-essential.

To test this hypothesis some other *erg* mutants were analyzed for endocytosis (Munn *et al*., 1999). Yeast cells require sterols for viability. Only the last steps of ergosterol biosynthesis (formation of zymosterol to ergosterol) are not essential and therefore enzymes that are involved in these steps can be mutated (see Figure 6). The *erg6Δ* and the double mutant *erg2Δ erg6Δ* strains were analyzed for α-factor uptake and LY accumulation at both 24°C and 37°C. The *erg6Δ* cells exhibited reduced internalization of α-factor at 24°C and 37°C and a wild-type LY accumulation in the vacuole, whereas in the *erg2Δ erg6Δ* mutant cells both LY and α-factor uptake were completely defective (Munn *et al*., 1999). The *ERG6* gene encodes the sterol C-24 methyl transferase and catalyses the formation of fecosterol that serves as substrate for the Erg2p enzyme (see Figure 6; McCammon *et al*., 1984). One characteristic of the ergosterol biosynthesis pathway is that mutations in the Erg2p and/or Erg6p do not prevent subsequent enzymes from altering the improperly modified substrates (Lees *et al*., 1995; Parks and Casey, 1995). Thus these mutations lead to accumulation of sterol structures that are not normal intermediates of the pathway. To correlate the endocytic defects that are observed in *erg2*, *erg6* and *erg2 erg6* mutants with the sterol composition, the sterols accumulating in these different *erg* mutants were analyzed. The results suggested that the state of desaturation of the B-ring may be critical for the internalization step of endocytosis in yeast (Munn *et al*., 1999).

Recently, plasma membrane cholesterol was shown to play a critical role in clathrin-coated pit internalization in mammalian cells (Rodal *et al*., 1999; Subtil *et al*., 1999). Cholesterol depletion from the plasma membrane inhibited transferrin and EGF endocytosis, demonstrating an essential role of cholesterol for the formation of clathrin-coated endocytic vesicles. In summary, sterols were identified as a novel requirement for endocytosis in yeast and in animal cells, but the exact role(s) of sterols are unknown up to now. The use of genetic approaches should help to clarify the ergosterol requirement for endocytosis in yeast.

6. Outlook

The studies in yeast have revealed several crucial aspects of the internalization step of receptor-mediated endocytosis. The actin cytoskeleton plays a major role and several actin-associated protein complexes are involved in the process. A great number of proteins have been isolated based on their role in the internalization step of endocytosis and surely more proteins will be discovered in the future. However, very little is known about the function of these proteins in endocytosis. The Arp2/3 complex has been implicated in actin-nucleation and pointed-end capping but whether these functions are required for endocytosis or if there is an additional endocytic function of the complex is unknown. The type I myosin, Myo5p, is a motor protein and thus may be involved in force generation in endocytosis. However, nothing is known about which step(s) in the endocytic pathway require force and how this force is generated. The major goals for the future are to understand the precise role of the different protein complexes involved in the internalization step, how their functions are regulated and how they co-operate to drive the internalization step of endocytosis.

The lipid requirement for endocytosis in yeast is still conceptually at an early stage. The link between a sphingosine signaling pathway and the internalization step of endocytosis remains to be defined. The near future should provide significant advances in the identification and characterization of downstream effectors and upstream regulators of the sphingosine

activated signaling pathway. Likewise, there is a gap in our understanding of the ergosterol requirement for the internalization step. Analysis of other viable *erg* single and double mutants should permit the development of theories to explain how sterols function in endocytosis.

The combination of genetic and biochemical approaches should help to answer the remaining questions about the protein and lipid requirements in yeast endocytosis.

7. Acknowledgments

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Recent findings

The internalization step of endocytosis is differentially affected by different actin mutant alleles. The findings that two actin mutant alleles (*act1-1*, *end7-1*) affect the internalization step of endocytosis (Kübler and Riezman, 1993; Munn *et al*., 1995) and that most other internalization mutants (e.g. *sla2/end4*, *rvs167*) affect the actin cytoskeleton, clearly support a central role of actin in endocytosis in yeast. However, the actin requirement seems to be rather specific since other mutants disrupting proper actin localization (e.g. *pfy1*, *tpm1*) do not affect endocytosis (Munn *et al*., 1995). A recent study has further investigated the role of actin in endocytosis by using an isogenic collection of actin mutants (Whitacre *et al*., 2001). The actin mutants exhibited a wide range in their abilities to internalize α-factor at 25°C and 37°C. Some alleles internalize like a wt strain or are defective at both temperatures, while other alleles have a *ts* phenotype with defects in internalization only at 37°C. Interestingly, there may be a correlation between defects in internalization and growth on salt of the different actin alleles with the exception of *act1-4*. The *act1-4* allele is severly impaired for internalization at 25°C and 37°C but is not especially salt-sensitive suggesting that this allele has a somewhat more specific effect on endocytosis (Whitacre *et al*., 2001). Furthermore, the same study demonstrated that internalization in yeast is affected by high external osmolarity (600 mM NaCl or 1 M sorbitol) as is the case in mammalian cells.

1. New proteins involved in endocytosis

A recent study has used a mutant library generated by the European Functional Analysis Network (EUROFAN) to screen for strains defective in fluid-phase endocytosis. A total of 700 strains disrupted for a single non-essential ORF were tested and 14 mutants were identified (Wiederkehr *et al*., 2001). As about 2% of the tested strains were defective in the accumulation of LY in the vacuole, the authors extrapolated that approximately 110 non-essential genes present in the yeast genome might be required for efficient fluid phase endocytosis. Interestingly, only 2 of the 14 mutants show a partial defect in the internalization step (Gagny *et al*., 2000; Wiederkehr *et al*., 2001) while the rest was affected in post-internalization steps. This is in marked contrast to previous screens for endocytosis-deficient mutants which yielded mostly internalization mutants (see D'Hondt *et al*., 2000, and references therein). Both new internalization mutants, *YNL177c* and *EDE1*, show a mild defect affecting mostly the initial internalization rate. Interestingly, Ede1p contains several EH-domains like two other proteins (End3p and Pan1p) already implicated in the internalization step (Benedetti *et al*., 1994; Raths *et al*., 1993; Tang *et al*., 1997; Wendland *et al*., 1996). Combination of *ede1Δ* with either *rsp5-1*, *pan1-10* or *end3-1* leads to a synthetic growth defect (Gagny *et al*., 2000) suggesting that Ede1p might be linked to the Pan1p-complex previously described (see section 3.2.4).

2. Actin-associated protein complexes

Recent studies have found an increasing cross-talk between the previously described protein complexes involved in endocytosis (see section 3.2). Interestingly, several of these findings deal with the Arp2/3 complex further emphasizing its central role in actin dynamics.

As mentioned previously, Cmd1p has at least two roles in endocytosis. One is to regulate the type I myosins Myo3p and Myo5p, and this function is affected by the *cmd1-247* and *cmd1-226* mutations. The second function is affected by the *cmd1-228* and *cmd1-226* mutations and the target is unknown (Geli *et al*., 1998). A recent study identified Arc35p, the 35 kDa subunit of the Arp2/3 complex, as the second target of Cmd1p in endocytosis (Schaerer-Brodbeck and Riezman, 2000a). Overexpression of wild type or mutant Cmd1p (except cmd1-228p and cmd1-226p) suppressed both the endocytic and actin cytoskeleton defect of *arc35-1*. Furthermore, Arc35p was shown to interact with Cmd1p and to be required for the cortical localization of Cmd1p (Schaerer-Brodbeck and Riezman, 2000a). Taken together these data suggest an important functional interaction between the 35 kDa subunit of the Arp2/3 complex Arc35p and calmodulin. Interestingly, the other target of Cmd1p function in endocytosis, the type I myosins (Geli *et al*., 1998), has been implicated in Arp2/3 complex mediated actin assembly (Evangelista *et al*., 2000; Lechler *et al*., 2000; see section 4 of the Results). The acidic domain of type I myosins interacts with the Arp2/3 complex via Arc40p and Arc19p. Furthermore, Las17p also interacts with Myo3/5p. More importantly, the acidic domains of Myo3/5p were shown to be genetically redundant with the acidic domain of Las17p (Evangelista *et al*., 2000; Lechler *et al*., 2000). These findings explain the mild phenotype of deleting the acidic domain of Las17p compared to a complete knockout *las17Δ* (Winter *et al*., 1999), since Myo3/5p are still present. Using an *in vitro* assay, Myo3/5p were shown to be essential for cortical actin assembly. This function required motor activity as well as phosphorylation of the motor domain by PAK's (Lechler *et al*., 2000). Interestingly, the function of Myo5p in actin polymerization requires interaction with Vrp1p (see section 4 of the Results). Taken together these findings support a very close connection between the Myo5p complex and the Arp2/3 complex (see section 3.2.).

Abp1p has been identified as another activator of Arp2/3 complex (Goode *et al*., 2001). Abp1p interacts biochemically with the Arp2/3 complex and purified Abp1p activates the actin polymerization activity of the Arp2/3 complex *in vitro* (Goode *et al*., 2001). Abp1p has been shown to interact with Rvs167p (Lila and Drubin, 1997) and might therefore connect the Rvs167p complex and the Arp2/3 complex (see section 3.2.).

A recent two-hybrid screen using Rvs167p and Rvs161p as baits identified a large number of interacting proteins (Bon *et al*., 2000). However, since no confirmation of the interactions using other techniques were made, these proteins cannot be considered bona fide interactors. Instead, a possible interesting link between the Rvs167p complex and the Pan1p complex via Sla1p might take place (Ayscough *et al*., 1999; Tang *et al*., 2000). Sla1p was shown to associate with both Pan1p and End3p (Tang *et al*., 2000) suggesting that Sla1p participates in the Pan1p complex. Another study showed that Sla1p is required for correct localization of Sla2p (Ayscough *et al*., 1999), a component of the Rvs167p complex, suggesting that Sla1p might regulate Sla2p function.

In summary, these recent findings point to a complex protein-protein interactions network involved in regulating actin dynamics and/or endocytosis in yeast.

3. Sphingoid base requirement

The *lcb1-100* (*end8-1*) mutant was isolated in a screen for endocytosis-deficient mutants and shown to affect the internalization step (Munn and Riezman, 1994). Since Lcb1p is involved in the first step of sphingolipid biosynthesis, these findings suggested that ongoing sphingoid base synthesis is required for endocytosis. Indeed, exogenous sphingoid bases can suppress the endocytic defect of *lcb1-100* (Zanolari *et al*., 2000). In order to identify the intermediate in the sphingolipid synthesis pathway required for the internalization, the *lcb1-100* mutant was combined with mutations that affect the utilization of exogenously added sphingoid bases. Using conditions, where no DHS-1-P (*lcb1/4/5*) or no ceramides (*lcb1/3/ysr3* respectively addition of australifungin) are made, addition of DHS still suppressed the internalization defect of *lcb1-100* (Zanolari *et al*., 2000). These data strongly suggest that sphingoid bases DHS or PHS are required for the internalization step of endocytosis in yeast. Interestingly, the *lcb1-100* strain shows a non-polarized actin cytoskeleton at 37°C. Addition of DHS can partially restore proper actin localization (Zanolari *et al*., 2000) suggesting that at least one function of sphingoid bases in endocytosis might be mediated via the actin cytoskeleton.

Since sphingoid bases are rapidly turned over, they are ideal candidates to act as signaling molecules. Indeed, inactivation of the *CDC55* regulatory or *PPH* catalytic subunits of protein phosphatase 2A (PP2A) suppress the endocytic defect of *lcb1-100* (Friant *et al*., 2000). Similarly, overexpression of the protein kinases Yck2p (completely) or Pkc1p (partially) suppressed the endocytic defect of *lcb1-100* (Friant *et al*., 2000). These data suggest a function of sphingoid bases in activating a protein phosphorylation pathway required for the internalization step. Interestingly, Yck2p and Pkc1p have an overlapping function in endocytosis because only cells with impaired activity of both kinases are defective for endocytosis (Friant *et al*., 2000). In addition, PP2A mutations or kinase overexpression partially suppressed the actin cytoskeleton defect of *lcb1-100* further supporting the hypothesis that the actin cytoskeleton is likely to be one target of sphingoid base synthesis requirement (Friant *et al*., 2000).

4. Sterols function early in Ste2p internalization

The *erg2-1* (*end11-1*) mutant was isolated in a screen for endocytosis-deficient mutants and shown to affect the internalization step (Munn and Riezman, 1994). Erg2p is involved in one of the late steps of ergosterol biosynthesis suggesting a function for sterols in endocytosis. A study using different *erg* mutants indicated that the state of desaturation of the B-ring may be important for the internalization step (Munn *et al*., 1999). However, a recent study analyzing more *erg* mutants has suggested that B-ring desaturation is not the sole structural requirement but that also side-chain methylation is important for the internalization step (Heese-Peck et al., submitted). In contrast to most other endocytosis mutants, at least two *erg* mutants (*erg2Δ erg6Δ*, *erg3Δ erg6Δ*) affecting internalization do not have pronounced actin cytoskeleton defects. However, they affect Ste2p phosphorylation (Heese-Peck et al., submitted) suggesting that sterols function early in receptor-mediated endocytosis in yeast. Interestingly, an *erg3*Δ strain internalizes α-factor like a wt strain while it is defective in fluid-phase endocytosis (LY-uptake) suggesting that sterols also function further downstream of the endocytic pathway on the way to the vacuole (Heese-Peck et al., submitted).

One possible role of sterols in endocytosis may be to provide the proper environment ("rafts") to recruit the endocytic machinery and/or cargo (e.g. Ste2p). Interestingly, two recent studies have demonstrated that homo-oligomeric complexes of Ste2p are functional units of endocytosis (Overton and Blumer, 2000; Yesilaltay and Jenness, 2000). A tempting hypothesis would be that Ste2p oligomerization requires a proper membrane environment (and thus appropriate sterols) and that oligomerization is required for efficient phosphorylation of the receptor. However, no effect of oligomerization of Ste2p on its own phosphorylation has been reported up to now.

Results

The results are presented in the form of four manuscripts. Two of them have been published and the other two are being submitted for publication.

Rvs161p and Rvs167p, the two yeast amphiphysin homologs, function together *in vivo*

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Abstract:

Mutations in *RVS161* and *RVS167*, the two yeast amphiphysin homologs, cause very similar growth phenotypes, a depolarized actin cytoskeleton and a defect in the internalization step of endocytosis. Rvs161p and Rvs167p have been shown to interact in the two-hybrid system but their localization in the cell may be different raising the question whether the interaction is physiologically relevant. Here we demonstrate that the two proteins function together *in vivo*. We find that the steady state level of Rvs167p is strongly reduced in a *rvs161Δ* strain. Similarly, the level of Rvs161p is strongly reduced in a *rvs167Δ* strain. We demonstrate that these reduced protein levels at steady state are due to a decreased stability of either Rvs protein in the absence of the other. Furthermore, we find that the amount and ratio of Rvs161p and Rvs167p are critical parameters for receptor-mediated endocytosis. In addition, using the two-hybrid system we show that the interaction of Rvs167p with actin is not abolished in an *abp1Δ* strain suggesting that Abp1p is not essential for this interaction.

I wrote the manuscript and performed all the experiments.

Introduction

The yeast Rvs161p and Rvs167p, together with mammalian amphiphysin I and II, nematode and fission yeast isoforms, constitute a family of conserved proteins (Wigge and McMahon, 1998). The N-termini of the different proteins share the highest homology and this common domain was called the BAR-domain (BIN/Amphiphysin/RVS-domain; Sakamuro *et al*., 1996). Rvs161p consists only of the BAR-domain (Figure 1) while the other members of the family have an SH3-domain at their C-termini and a central domain varying among the different proteins. In the case of Rvs167p, the central domain is rich in glycine, proline and alanine and therefore is called the GPA-domain (Figure 1).

Figure 1. Schematic overview of the domains of Rvs161p and Rvs167p
BAR, BIN/Amphiphysin/RVS domain; GPA, glycine-proline-alanine-rich region; SH3, Src homology 3 domain.

The mammalian homolog, amphiphysin I, was first identified as a brain protein enriched at presynaptic regions (Lichte *et al*., 1992). The identification of dynamin, synaptojanin, the α_c-subunit of AP2-adaptin and clathrin as amphiphysin I-interacting proteins further implicated amphiphysin I in endocytosis (Wigge and McMahon, 1998). A more broadly expressed isoform, amphiphysin II, has been identified by several groups and was found to interact with the same proteins that interact with amphiphysin I (Wigge and McMahon, 1998). Interestingly, the two isoforms can be coimmunoprecipitated from brain extracts suggesting that they act in concert (Wigge *et al*., 1997). Indeed, two studies (Ramjaun *et al*., 1997; Wigge *et al*., 1997) found that the two amphiphysin isoforms colocalize in brain. However, a third study identified a different subcellular localization of the two isoforms (Butler *et al*., 1997). Amphiphysin I was shown to be concentrated in the cortical cytoplasm of nerve terminals, whereas amphiphysin II was concentrated in axon initial segments and nodes of Ranvier (Butler *et al*., 1997).

The two yeast members of the amphiphysin family, encoded by *RVS161* and *RVS167*, were first identified in a screen for mutations causing reduced viability upon nutrient starvation (Bauer *et al*., 1993; Crouzet *et al*., 1991). *RVS161* was also identified in a screen for endocytosis mutants (Munn *et al*., 1995). Mutations in *rvs161* or *rvs167* exhibit the same phenotypes except for a defect in cell fusion only found in the *rvs161* mutant (Brizzio *et al*., 1998). The mutant phenotypes include defects in endocytosis, cell polarization, bud site selection in diploid cells, and a depolarized actin cytoskeleton (Bauer *et al*., 1993; Munn *et al*., 1995; Sivadon *et al*., 1995). Rvs161p and Rvs167p have been shown to interact through the BAR-domain in the two-hybrid system (Navarro *et al*., 1997). However, their localization in the cell seems to be different, raising the question whether the Rvs161p-Rvs167p interaction is relevant *in vivo*. Rvs161p was shown to be mainly cytosolic in unbudded cells and upon bud emergence it localizes mainly to the mother-bud neck region (Brizzio *et al*., 1998). In contrast, in unbudded cells Rvs167p is localized mainly in small cortical patches throughout the cell which polarize at the bud emergence site and in small buds (Balguerie *et al*., 1999). Using the two-hybrid system the BAR-domain of Rvs167 has also been shown to mediate homodimerization in one study (Colwill *et al*., 1999) while another study failed to detect any Rvs167p-Rvs167p interaction (Navarro *et al*., 1997).

Mutations in *rvs161* and *rvs167* affect the actin cytoskeleton (Bauer *et al*., 1993; Sivadon *et al*., 1995). Interestingly, the SH3-domain of Rvs167p has been shown to interact with actin in the two-hybrid system (Amberg *et al*., 1995). The finding that Rvs167p interacts with the actin binding protein Abp1p through its GPA/SH3-domains (Lila and Drubin, 1997) led to the hypothesis that Abp1p could mediate the interaction between Rvs167p and actin.

In this study we investigated whether Rvs161p and Rvs167p indeed function together *in vivo*. We find that the steady state level of Rvs161p is strongly reduced in a *rvs167Δ* strain. Similarly, the level of Rvs167p is strongly reduced in a *rvs161Δ* strain. We demonstrate that these reduced protein levels at steady state are caused by a decreased stability of either Rvs protein in a strain mutated in the other *rvs* gene. Furthermore, we provide evidence that the amount and ratio of Rvs161p and Rvs167p are critical parameters for receptor-mediated endocytosis. In addition, using the two-hybrid system we find that Abp1p is not required to mediate the interaction of Rvs167p with actin.

Material and Methods

Yeast strains, media and general techniques

Yeast strains used in this study were EGY48 (*Mat **a** his3 trp1 ura3 leu2::lexAop6-LEU2*), RH448 (*Mat **a** his4 leu2 lys2 ura3 bar1*), RH3376 (*Mat **a** his3 leu2 trp1 ura3 bar1*), RH2600 (*Mat **a** his4 ura3 rvs161Δ bar1*), RH2950 (*Mat **a** his4 leu2 trp1::URA3 ura3 rvs167::TRP1 bar1*), RH5238 (*Mat **a** his3 trp1 ura3 leu2::lexAop6-LEU2 abp1::KanMX6*) and RH5239 (*Mat **a** his3 trp1 ura3 leu2::lexAop6-LEU2 rvs161::KanMX6*). Strains that did not bear plasmids were grown in complete media YPUAD (2% glucose, 2% peptone, 1% yeast extract, 40 μg/ml uracil, 40 μg/ml adenine and 40 μg/ml tryptophan, 2% agar for solid media). Unless mentioned otherwise, strains bearing plasmids were selected on SD minimal media (Dulic *et al*., 1991). Standard recombinant DNA techniques were used (Sambrook *et al*., 1989). Restriction endonucleases were obtained from MBI Fermentas, Klenow from Boehringer Mannheim and Pfu polymerase was obtained from Stratagene.

Plasmid constructions

pEG202 and pJG4-5 were described elsewhere (Gyuris *et al*., 1993), both plasmids contain a *2μ* origin of replication. The reporter gene plasmid pSH18-34 was described elsewhere (Estojak *et al*., 1995), it contains eight LexA-operators upstream of the reporter gene *GAL1*-LacZ. To construct pEG*RVS161* the *RVS161*-ORF was amplified by the polymerase chain reaction with Pfu polymerase using the 5'-primer GATACGGAATTCATGAGTT GGGAAGGTTTTAAG and 3'-primer GAGTATTCCGCTCGAGTTATTTTATC CCGAGCGCACAAAT introducing an EcoR I-site upstream and a Xho I-site downstream of the *RVS161*-ORF. The fragment was then inserted as an EcoR I-Xho I-fragment into pEG202. To construct pJG*ACT1* the *ACT1*-ORF was amplified by the polymerase chain reaction using the 5'-primer GATACGGAATTCATGGATT CTGGTATGTTCTAG and 3'-primer TACGCGGATCCTTAGAAACACTTGT GGTGAACG introducing an EcoR I-site upstream and a BamH I-site downstream of the *ACT1*-ORF. The fragment was then cut with BamH I, the 5' overhang filled in with Klenow, and cut with EcoR I. This fragment was then inserted in a pJG4-5 which was cut with Xho I, the 5' overhang filled in with Klenow, and cut with EcoR I. To construct pEG*RVS167* the *RVS167*-ORF was amplified by the polymerase chain reaction using the 5'-primer

RVS167,1 (GATACGGAATTCATGAGT TTTAAAGGGTTTACCAAG) and 3'-primer RVS167,2 (GAGTATTCCGCT CGAGCTAGTTCTTGTTGAGTTGCAC G) introducing an EcoR I-site upstream and a Xho I-site downstream of the *RVS167*-ORF. The fragment was then inserted as an EcoR I-Xho I-fragment into pEG202. pJG*RVS167* was described elsewhere (Geli *et al*., 2000). To construct pJGBAR the BAR-domain of *RVS167* was amplified by the polymerase chain reaction using the 5'-primer RVS167,1 and 3'-primer GAGTATTCCGCTCGAGCTA CTTAGAGTAACCGAGTTTAAAG introducing an EcoR I-site upstream and a Xho I-site downstream of the BAR-domain. The fragment was then inserted as an EcoR I-Xho I-fragment into pJG4-5. To construct pJGBAR/GPA the BAR- and GPA-domains of *RVS167* were amplified by the polymerase chain reaction using the 5'-primer RVS167,1 and 3'-primer RVS167,6 (GAGTATTCCGCTCGAGCT AGCCAGGAGCTGCCGCTACG) introducing an EcoR I-site upstream and a Xho I-site downstream of the BAR/GPA-domains. The fragment was then inserted as an EcoR I-Xho I-fragment into pJG4-5. To construct pJGGPA the GPA-domain of *RVS167* was amplified by the polymerase chain reaction using the 5'-primer RVS167,8 (GATACGGAATTCCTTTAA ACTCGGTTACTCTAAG) and 3'-primer RVS167,6 introducing an EcoR I-site upstream and a Xho I-site downstream of the GPA-domain. The fragment was then inserted as an EcoR I-Xho I-fragment into pJG4-5. To construct pJGGPA/SH3 the GPA-and SH3-domains of *RVS167* were amplified by the polymerase chain reaction using the 5'-primer RVS167,8 and 3'-primer RVS167,2 introducing an EcoR I-site upstream and a Xho I-site downstream of the GPA/SH3-domains. The fragment was then inserted as an EcoR I-Xho I-fragment into pJG4-5. To construct pJGSH3 the SH3-domain of *RVS167* was amplified by the polymerase chain reaction using the 5'-primer GATACGGAATTCCCTGGCGTTGAAA CTGTTACCG and 3'-primer RVS167,2 introducing an EcoR I-site upstream and a Xho I-site downstream of the SH3-domain. The fragment was then inserted as an EcoR I-Xho I-fragment into pJG4-5. To construct p181*RVS161* and p195*RVS161* the 2.2-kb EcoR I fragment from pUB1-3 (Munn *et al*., 1995) was excised and cloned into Yeplac181 (p181; Gietz and Sugino, 1988) and Yeplac195 (p195; Gietz and Sugino, 1988). To construct p181*RVS167* and p195*RVS167* the 1.8-kb Kpn I-Sph I fragment from pFBKS (Bauer *et al*., 1993) was excised and cloned into Yeplac181 (p181) and Yeplac195 (p195).

Two-hybrid analysis

Two-hybrid analysis was performed as described (Geli *et al*., 1998). Briefly, the strains contain the reporter plasmid pSH18-34 with the reporter gene *GAL1*-LacZ under the control of eight LexA-operators. To assay the interaction between bait and prey the strains are streaked out on plates containing X-Gal. The positive colonies turn dark while the negative ones remain white.

Protein extracts and immunoblotting

Yeast strains were grown to exponential phase, harvested and lysed with glass beads in 500 µl lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% SDS and 1 µg/ml of the protease inhibitors aprotinin, leupeptin, pepstatin A, chymostatin and antipain). The protein concentration of the lysates was measured using the Bio-Rad protein assay. Equivalent amounts of each sample were analyzed by SDS-PAGE (Laemmli, 1970) and immunoblotting with antibodies against Rvs161p and Rvs167p. As a control, antibodies against Sla2p were used (Wesp *et al*., 1997).

Pulse-chase experiments

Metabolic labeling of cells was carried out as described previously (Munn *et al*., 1995) with the modification that the cells were not converted to spheroplasts, but were lysed with glass beads in 200 µl of lysis buffer and then heated for 3 minutes at 95°C. Rvs161p or Rvs167p were immunoprecipitated from the lysates and analyzed by SDS-PAGE and autoradiography.

Coimmunoprecipitation

WT (RH448) and *rvs167Δ* (RH2950) strains were grown to exponential phase, harvested and converted to spheroplasts using lyticase (Raths *et al*., 1993). The spheroplasts were lysed by osmotic shock in 2 ml of buffer (20 mM MES pH 6.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 0.5 mM PMSF and 1 µg/ml of the protease inhibitors pepstatin, leupeptin, antipain). The protein concentration of the lysates was measured using the Bio-Rad protein assay. Equal amounts of total protein for both strains were taken and proteins were immunoprecipitated with antibodies against Rvs167p. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with antibodies against Rvs161p.

α-factor uptakes

α-factor uptakes (continuous presence) were done as described previously (Dulic *et al*., 1991). The strains were tested at 24°C. The internalization rates were calculated as the percentage of counts internalized per time unit in the linear range and normalized to the internalization rate of the strain with the empty vector set to 100%. The results represent the average of at least three independent experiments and the standard deviation was calculated.

Growth curves

The strains transformed with the indicated plasmids were grown overnight and the cell density was determined. The cultures were diluted to about 10^6 cells/ml, a sample was taken (time point 0) and the cultures were incubated at 24°C. Every 3 hours a sample was taken and the cell density determined.

Actin staining

Yeast cell pre-cultures were grown at 24°C in SD selective media in order to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD to early log phase. Cells at 1.5×10^7 were then fixed in fomaldehyde and stained with TRITC-phalloidin (Sigma, St. Louis, MO) to visualize F-actin essentially as described previously (Benedetti *et al*., 1994).

Results

Interactions of the BAR-domain of Rvs167p

Rvs167p can be divided into three regions: the N-terminal BAR-domain, the GPA-domain and the C-terminal SH3-domain (Figure 1). Two previous studies have shown that the BAR-domain of Rvs167p interacts with Rvs161p. However, in one of these reports the BAR-domain of Rvs167p also interacted with full-length Rvs167p (Colwill *et al*., 1999) while in the other report no such interaction was detected (Navarro *et al*., 1997). In order to clarify this point we tested these interactions in a different two-hybrid system. Both previous studies used a two-hybrid system with the yeast Gal4p as DNA-binding domain while in our system the *E.coli* protein LexA is used as DNA-binding domain. As expected we detected a strong interaction of

Rvs161p with the BAR-domain of Rvs167p (Figure 2A). As shown in Figure 2B we also detected a strong interaction between full-length Rvs167p and the BAR-domain of Rvs167p. Since the BAR-domain of Rvs167p mediates both the interactions with Rvs161p and Rvs167p there is the possibility that the interaction of Rvs167p with itself is indirect and mediated via Rvs161p. To test this we performed a two-hybrid analysis in a *rvs161Δ* strain and still detected a strong Rvs167p-Rvs167p interaction (Figure 2C), suggesting that the interaction is direct or mediated by another protein than Rvs161p. To confirm the report that Rvs161p and Rvs167p form a complex *in vivo* (Navarro *et al*., 1997), we showed that the two proteins could be coimmunoprecipitated under native conditions with antibodies against Rvs167p (Figure 2D).

Reduced stability of Rvs proteins in the absence of its partner

Interestingly, the localization of Rvs161p (mostly cytoplasmic, at mother-bud neck region in small budded cells) and Rvs167p (small cortical patches that polarize upon bud emergence) seems to be different (Balguerie *et al*., 1999; Brizzio *et al*., 1998). These findings raise the question whether the interaction of Rvs161p with Rvs167p is required *in vivo*. We found that when compared to a WT strain at steady state, the level of Rvs167p is strongly reduced in a *rvs161Δ* strain (Figure 3A). Similarly the level of Rvs161p is strongly reduced in a *rvs167Δ* strain (Figure 3A). As control we detected Sla2p, a protein that is also required for endocytosis and actin organization (Wesp *et al*., 1997). Its levels were constant in the three strains (Figure 3A). A decreased synthesis or an increased instability of the proteins in the mutant strains could cause these reduced protein levels at steady state. In order to address this question, we performed pulse-chase experiments in the different strains. As shown in Figure 3B, both Rvs161p in a *rvs167Δ* strain and Rvs167p in a *rvs161Δ* strain are unstable when compared to a WT strain. Taken together these data suggest an *in vivo* function for the Rvs161p-Rvs167p interaction in stabilizing both proteins.

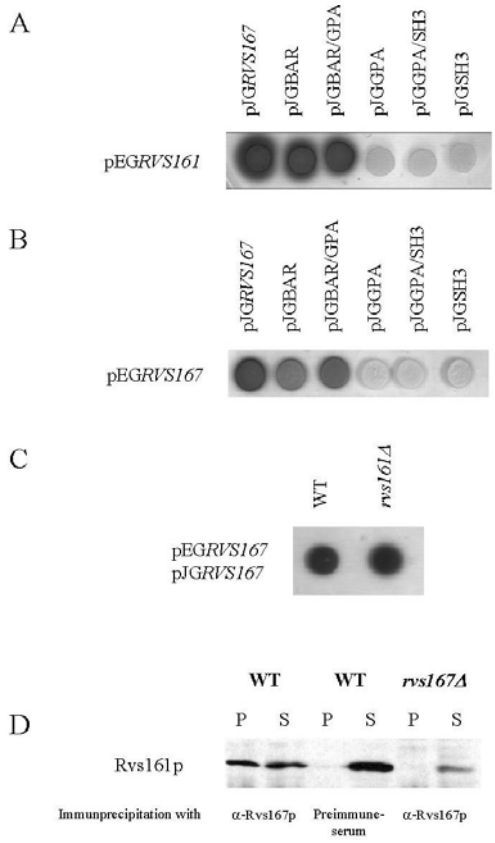

Figure 2. Two-hybrid interactions of the BAR domain of Rvs167p
Strains containing the reporter gene plasmid pSH18-34 with the LacZ-gene under the control of eight LexA-operators, a bait and a prey were streaked out on plates containing X-Gal. Positive interactors turn dark while negative colonies remain white. (A) Full-length Rvs161p as bait (pEG*RVS161*) was tested against full-length Rvs167p (pJG*RVS167*) and indicated Rvs167p-domains as preys. (B) Full-length Rvs167p as bait (pEG*RVS167*) was tested against full-length Rvs167p (pJG*RVS167*) and indicated Rvs167p-domains as preys. (C) The interaction of Rvs167p with itself was tested in a WT (EGY48) and in a *rvs161Δ* (RH5239) strain. (D) Coimmunoprecipitation of Rvs161p and Rvs167p. Lysates from WT (RH448) and *rvs167Δ* (RH2950) were incubated with antibodies against Rvs167p or with a preimmuneserum and the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with antibodies against Rvs161p.

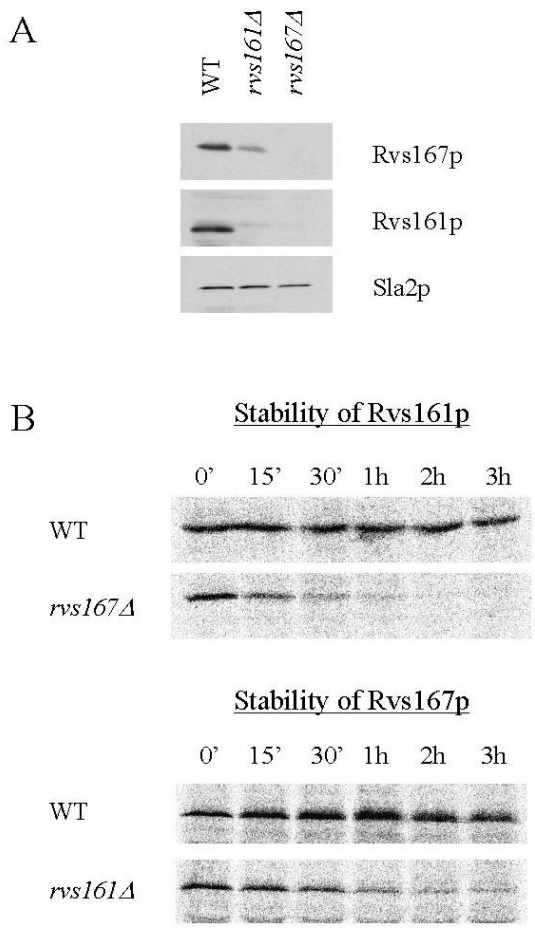

Figure 3. Interaction of Rvs161p and Rvs167p is required for the stability of both proteins
(A) Protein extracts from WT (RH3376), *rvs161Δ* (RH2600) and *rvs167Δ* (RH2950) at steady state were analyzed by SDS-PAGE and immunoblotting with antibodies against Rvs161p and Rvs167p. As a control the same extracts were blotted with antibodies against Sla2p. (B) Pulse-chase experiments. WT (RH3376), *rvs161Δ* (RH2600) and *rvs167Δ* (RH2950) cells were metabolically labeled and lysates were prepared as described in Materials and Methods. Proteins were immunoprecipitated from the lysates with antibodies against Rvs161p or Rvs167p and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

Amount and ratio of Rvs161p and Rvs167p are critical parameters for endocytosis

Mutations in *rvs161* or *rvs167* have both been shown to affect the internalization step of receptor-mediated endocytosis (Munn *et al*., 1995). In order to learn more about the involvement of the proteins in endocytosis we decided to overexpress them and measure how they affect α-factor internalization. Introduction of a *2μ*-plasmid with *RVS161* or *RVS167* in a WT strain does not affect α-factor internalization (Figure 4A). Interestingly, overexpression of Rvs167p in a *rvs161Δ* strain exacerbated the endocytic defect of this strain (Figure 4B). Similarly, overexpression of Rvs161p in a *rvs167Δ* strain exacerbated the endocytic defect of this strain (Figure 4C). The protein levels of Rvs161p and Rvs167p upon overexpression in a WT or a mutated strain are similar (data not shown). Interestingly, co-overexpression of both Rvs161p and Rvs167p in a WT strain reduces α-factor internalization to about two thirds of WT rate (Figure 4D). Taken together these data suggest that not only the amounts of Rvs161p and Rvs167p, but also their ratio are critical parameters for endocytosis.

We have tested the same strains overexpressing the Rvs proteins for an actin and a growth phenotype. As shown in Figures 5A-H, overexpression of the Rvs proteins either individually or together in a WT strain does not affect the actin cytoskeleton profoundly. The cells have a normal polarized actin cytoskeleton with cables running along the mother-bud axis. Also the growth rates of the different strains are not greatly affected (Figure 5I).

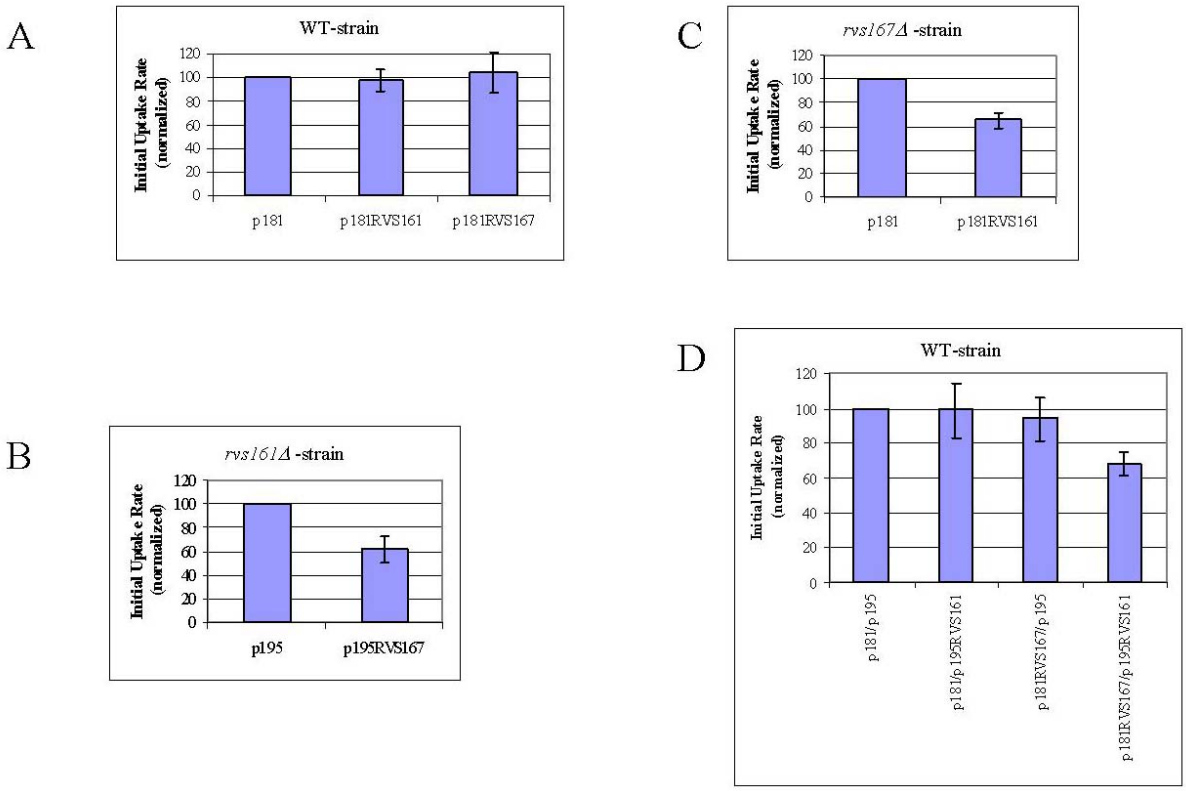

Figure 4. α-factor uptakes
(A) WT strain (RH3376) transformed with either p181, p181*RVS161* or p181*RVS167* were tested for α-factor internalization at 24°C. The internalization rates were calculated as the percentage of counts internalized per time unit in the linear range and normalized to the strain with p181 set to 100%. Note that the internalization rate of the WT strain transformed with p181 is ~ 4.6 %/min. (B) *rvs161Δ* strain (RH2600) transformed with either p195 or p195*RVS167* were tested for α-factor internalization at 24°C. The internalization rates were calculated as the percentage of counts internalized per time unit in the linear range and normalized to the strain with p195 set to 100%. Note that the internalization rate of the *rvs161Δ* strain transformed with p195 is ~1.7 %/min. (C) *rvs167Δ* strain (RH2950) transformed with either p181 or p181*RVS161* were tested for α-factor internalization at 24°C. The internalization rates were calculated as the percentage of counts internalized per time unit in the linear range and normalized to the strain with p181 set to 100%. Note that the internalization rate of the *rvs167Δ* strain transformed with p181 is ~ 2.0 %/min. (D) WT strain (RH3376) transformed with combinations of either p181, p195, p181*RVS167* and p195*RVS161* were tested for α-factor internalization at 24°C. The internalization rates were calculated as the percentage of counts internalized per time unit in the linear range and normalized to the strain with p181/p195 set to 100%.

As shown in Figures 6A to 6H, over-expression of Rvs167p in a *rvs161Δ* strain or overexpression of Rvs161p in a *rvs167Δ* strain does not further deteriorate or ameliorate the actin defect exhibited by the mutants. We detect a mild growth phenotype upon overexpression of Rvs167p in the *rvs161Δ* strain when compared to the strain with the empty vector (Figure 6I). Overexpression of Rvs161p in a *rvs167Δ* strain has no obvious effect on the growth rate (Figure 6I).

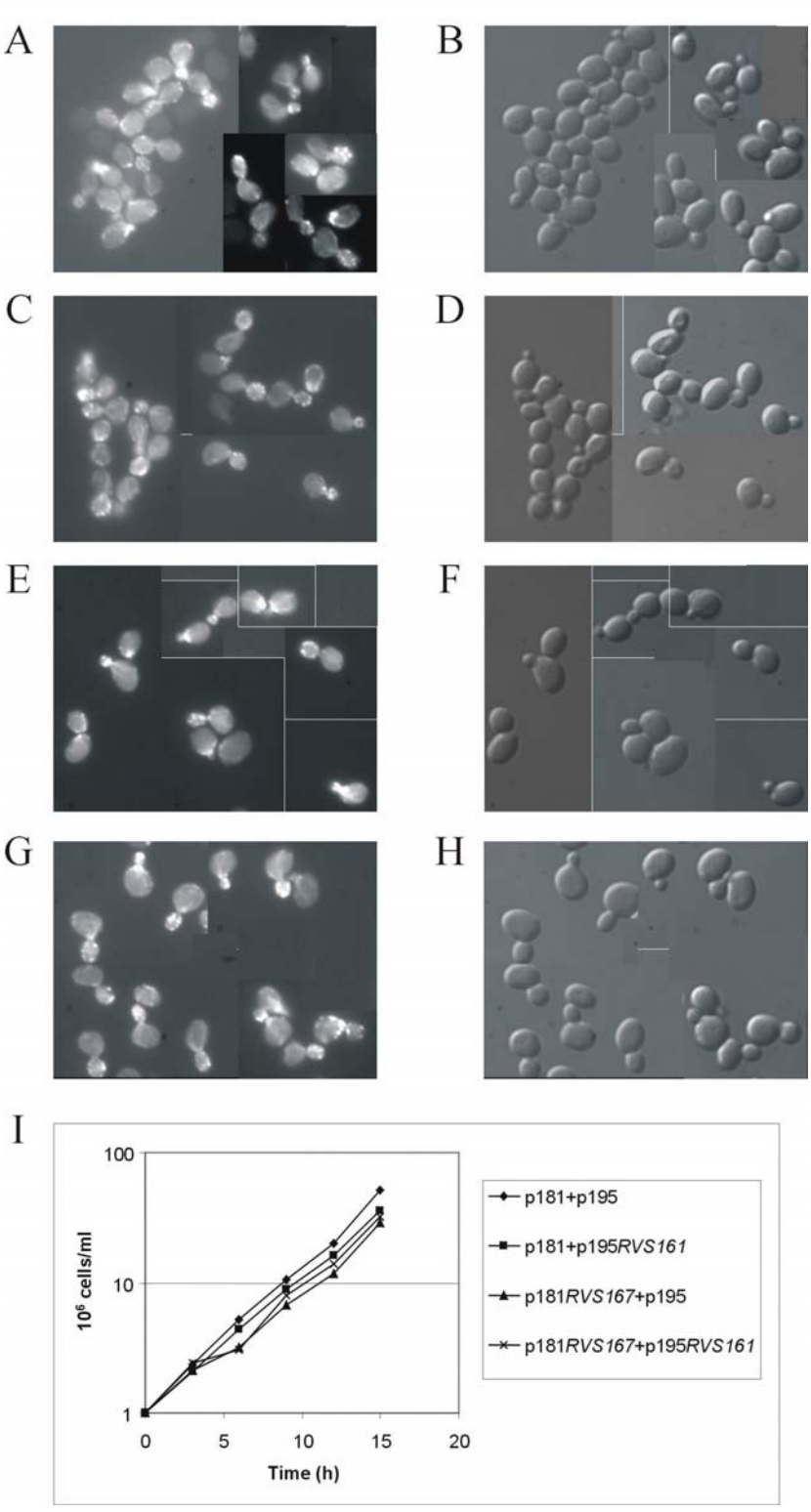

Figure 5. Actin staining and growth curve
(A - H) WT strain (RH3376) transformed with p181 and p195 (A, B), p181 and p195*RVS161* (C, D), p181*RVS167* and p195 (E, F), p181*RVS167* and p195*RVS161* (G, H) were fixed and filamentous actin was visualized using TRITC-phalloidin (panels A, C, E, G) or Nomarski optics (panels B, D, F, H). (I) The same strains were grown overnight. The cultures were diluted to about 10^6 cells/ml, incubated at 24°C and the cell densities were determined every three hours.

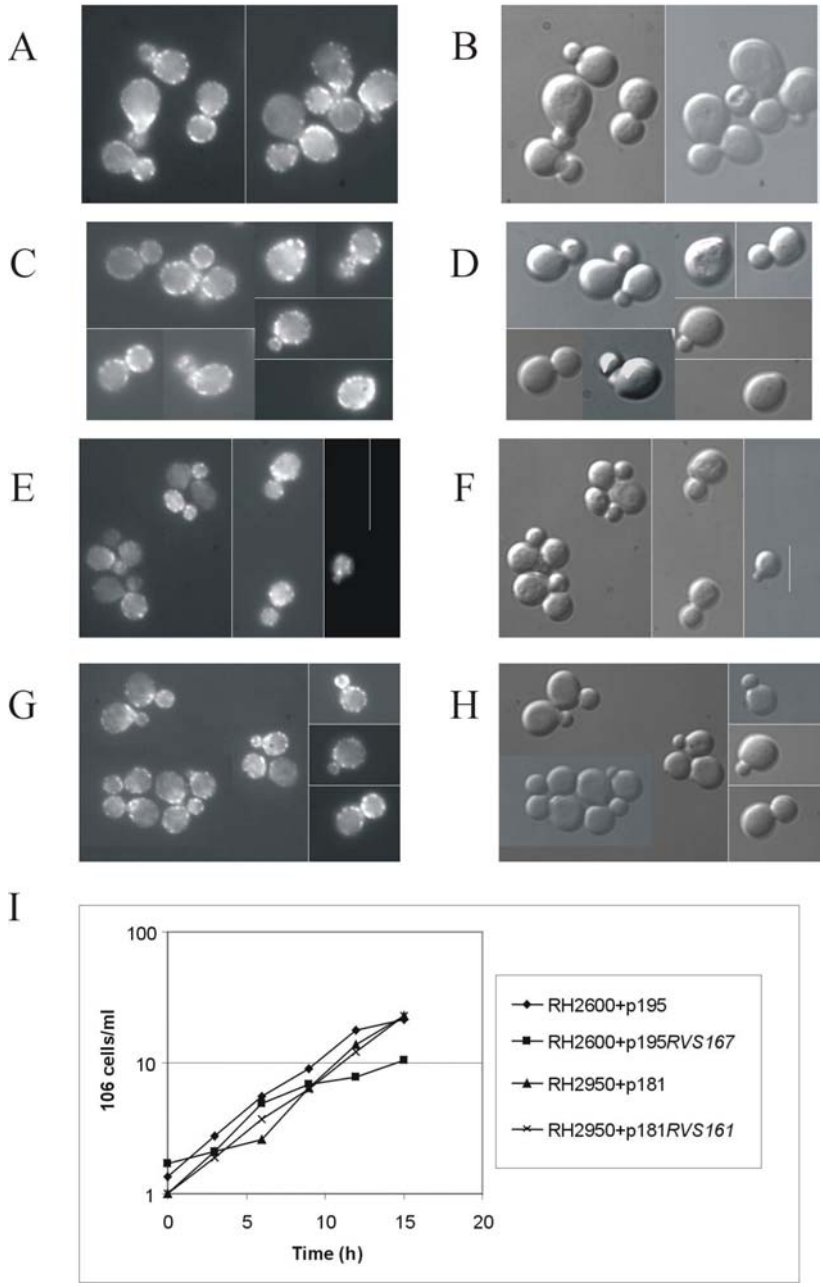

Figure 6. Actin staining and growth curve
(A - D) *rvs161Δ* strain (RH2600) transformed with either p195 (A, B) or p195*RVS167* (C, D) were fixed and filamentous actin was visualized using TRITC-phalloidin (panels A, C) or Nomarski optics (panels B, D). (E - H) *rvs167Δ* strain (RH2950) transformed with either p181 (E, F) or p181*RVS161* (G, H) were fixed and filamentous actin was visualized using TRITC-phalloidin (panels E, G) or Nomarski optics (panels F, H). (I) The same strains were grown overnight. The cultures were diluted to about 10^6 cells/ml, incubated at 24°C and the cell densities were determined every three hours.

Interaction of Rvs167p with actin does not require Abp1p

Mutations in *rvs167* affect the actin cytoskeleton (Bauer *et al*., 1993) and Rvs167p has been shown to interact with actin via its SH3-domain (Amberg *et al*., 1995). The actin binding protein Abp1p has been proposed to mediate this interaction of Rvs167p and actin since it interacts with the GPA/SH3-domains of Rvs167p (Lila and Drubin, 1997). As

shown in Figure 7, the interaction of Rvs167p with actin in the two-hybrid system is not abolished in an *abp1Δ* strain showing that Abp1p is not required to mediate the interaction of Rvs167p with actin. Interestingly, we also detect an interaction of Rvs161p with actin in the two-hybrid system. However, this interaction is abolished in a *rvs167Δ* strain suggesting that the interaction of Rvs161p with actin is mediated via Rvs167p (data not shown).

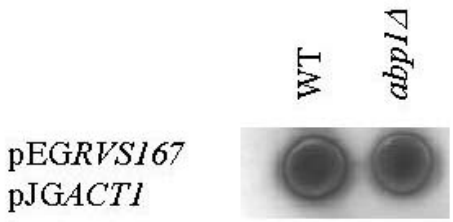

Figure 7. Two-hybrid analysis
The interaction of Rvs167p (pEG*RVS167*) with actin (pJG*ACT1*) was tested in a WT (EGY48) and in an *abp1Δ* (RH5238) strain. The strains contain the reporter gene plasmid pSH18-34 with the LacZ-gene under the control of eight LexA-operators, a bait and a prey. They were streaked out on plates containing X-Gal. Positive interactors turn dark while negative colonies remain white.

Discussion

In this study we provide direct evidence that Rvs161p and Rvs167p function together *in vivo*. We find that the steady state levels of the two proteins are inderdependent. This effect is caused by a dramatically decreased stability of either Rvs protein in the absence of its partner. These data provide evidence that the interaction of Rvs161p and Rvs167p is physiologically relevant and required for the stability of both proteins. Furthermore these findings might explain the almost identical phenotypes that are seen upon mutation of the two genes individually. The mutant phenotypes detected in either mutant strain might be a combination of loss of both Rvs proteins. Nevertheless, one function, the Rvs161p function in cell fusion (Brizzio *et al*., 1998), does not seem to require Rvs protein-protein interaction. Apparently, the highly reduced levels of Rvs161p in the *rvs167Δ* mutant are sufficient for this function. As seen in Figure 3B, Rvs161p is more sensitive to Rvs167p levels than vice versa. Since Rvs167p has two additional domains when compared to Rvs161p this difference might reflect a partial stabilization of Rvs167p in the absence of Rvs161p via interactions mediated by these domains with other proteins (e.g. interaction with actin).

As mentioned in the introduction, two previous studies have investigated the cellular localization of Rvs161p (mostly cytoplasmic, at mother-bud neck region in small budded cells; Brizzio *et al*., 1998) and Rvs167p (small cortical patches that polarize upon bud emergence; Balguerie *et al*., 1999). The two proteins apparently do not co-localize in the cell, however in this study we have provided evidence that these two proteins do function together *in vivo*. This discrepancy might be explained if one considers that Rvs161p localization is mainly cytosolic. Therefore it might also be localized to the Rvs167p-containing patches but is not concentrated there. In the case of Rvs167p, the bright fluorescence caused by its high concentration in cortical patches might make it difficult to detect an additional weak diffuse cytosolic staining. Therefore it is possible, that a certain amount of Rvs167p is localized to the cytosol and interacts with Rvs161p.

Interestingly, overexpression of either Rvs161p or Rvs167p alone in a WT strain has no major effect on the internalization step of endocytosis. However, co-overexpression of both proteins reduced the α-factor internalization rate to two thirds of WT levels. Also, overexpression of Rvs161p in a *rvs167Δ* strain, as well as overexpression of Rvs167p in a *rvs161Δ* strain, exacerbated the endocytic defect

found in the mutated strains. Since the amount of protein upon overexpression is similar in all the strains (WT, *rvs161Δ* and *rvs167Δ*, data not shown) we conclude that both the amount and the ratio of Rvs161p and Rvs167p are critical parameters for the internalization step of endocytosis. We have tested the strains used for the endocytosis assays for an actin and a growth phenotype. None of the strains overexpressing the Rvs proteins exhibited an obvious change in their actin cytoskeleton when compared to the strains with the empty vectors. Also the growth rates were almost identical with the exception of a mild defect detected upon overexpression of Rvs167p in the *rvs161Δ* strain. The endocytic defect we detect in some of the strains overexpressing the Rvs proteins might be explained in two ways. One possibility would be that overexpression of the Rvs proteins causes a very subtle actin defect not detectable by immunofluorescence but affecting endocytosis. Another possibility would be that by overexpressing the Rvs proteins another protein required for endocytosis is titrated out and therefore endocytosis is affected.

By using the two-hybrid system we detected an interaction of Rvs167p with itself mediated via the BAR-domain as previously described (Colwill *et al*., 1999). Since the same domain also mediates the interaction with Rvs161p, we wanted to determine if the Rvs167p-Rvs167p interaction is direct or mediated via Rvs161p. Two-hybrid analysis in an *rvs161Δ* strain demonstrated that Rvs161p is not required for this interaction and therefore Rvs167p either interacts directly with itself or the interaction is mediated via another protein than Rvs161p. Interestingly, amphiphysin I has also been shown to interact with itself in mammalian cells (Slepnev *et al*., 1998).

Mutations in *rvs161* and *rvs167* have been shown to affect the actin cytoskeleton (Bauer *et al*., 1993; Sivadon *et al*., 1995). A previous study has shown that Rvs167p interacts with actin via its SH3-domain (Amberg *et al*., 1995) and it was suggested that the actin binding protein Abp1p could mediate this Rvs167p-actin interaction (Lila and Drubin, 1997). Here we show that in the two-hybrid system Rvs167p interacts with actin in an *abp1Δ* strain showing that Abp1p is not required for this interaction. There are several possible explanations for this finding. First, it could be that in the absence of Abp1p another protein takes over its function. Second, there could be more than one protein involved in mediating this interaction. Third, Rvs167p could interact directly with actin. Another potential candidate to mediate the Rvs167p-actin interaction could be Las17p, since it was shown to interact with the GPA/SH3 domains of Rvs167p in the two-hybrid system (Madania *et al*., 1999).

rvs mutant cells show pleiotropic phenotypes including sensitivity to non-optimal growth conditions, defects in the organization of the actin cytoskeleton, defects in bud-site selection and endocytosis (Bauer *et al*., 1993; Munn *et al*., 1995; Sivadon *et al*., 1995). Interestingly, Rvs167p was identified as a target of the Pho85 cyclin-dependent kinase and thus might be involved in the regulation of the actin cytoskeleton early in cell cycle (Lee *et al*., 1998). Also its mammalian homolog, amphiphysin, has been shown to be regulated by phosphorylation (Bauerfeind *et al*., 1997; Slepnev *et al*., 1998). In addition, amphiphysin I has been implicated in the autoimmune Stiff-Man syndrome disorder associated with breast cancer and amphiphysin II has been shown to interact with the MYC oncoprotein implicating it in

cell cycle control (David *et al*., 1994; Sakamuro *et al*., 1996). Taken together these data suggest that some general functions and regulation of these proteins have been conserved through evolution. In addition, these findings implicate the members of this protein family not only as important players in intracellular membrane trafficking but also in mediating signals during the cell cycle.

In summary, we demonstrate for the first time that the yeast amphiphysin homologs Rvs161p and Rvs167p function together *in vivo*. The interaction between the proteins seems to be crucial for their stability. Furthermore, we show that the amount and ratio of Rvs161p and Rvs167p are critical parameters for the internalization step of endocytosis.

Acknowledgments

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Sphingosine signaling pathway via Pkh1/2 kinases is required for endocytosis in yeast

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Abstract:

In yeast, sphingoid base synthesis is required for the internalization step of endocytosis and organization of the actin cytoskeleton. We show that overexpression of one of two kinases Pkh1p or Pkh2p, that are homologous to mammalian 3-phosphoinositide-dependent kinase-1 (PDK1), can specifically suppress the sphingoid base synthesis requirement for endocytosis. Pkh1p and Pkh2p have an overlapping function because only a mutant with impaired function of both kinases is defective for endocytosis. We show that Pkh1/2p kinases are activated *in vitro* by nanomolar concentrations of sphingoid base (PHS). These results suggest that Pkh1/2p kinases are part of a sphingoid base-mediated signaling pathway that is required for the internalization step of endocytosis. Two downstream effectors of this signaling cascade were identified, the Pkc1p kinase and the amphiphysin homolog, Rvs167p. Both proteins are phosphorylated by Pkh1/2p kinases and play a role in endocytosis.

My contribution to this work is shown in Figure 3A.

Introduction

Endocytosis is the process whereby eukaryotic cells internalize extracellular material as well as part of their own plasma membrane. This pathway is commonly used for uptake of nutrients, down-regulation of receptors and removal of other proteins from the cell surface. The development of several reporter systems to study endocytosis, as well as the use of genetic studies in the yeast *Saccharomyces cerevisiae* has allowed the identification of many components required for this pathway. The internalization step of endocytosis requires actin, proteins that are required for proper actin cytoskeleton organization, such as fimbrin, calmodulin, type I myosin, the amphiphysin homologs Rvs161p and Rvs167p, clathrin, and a large set of proteins associated to these major components (D'Hondt *et al*., 2000; Geli and Riezman, 1998; Lombardi *et al*., 2001; Riezman *et al*., 1996).

Recently, not only proteins but also lipids have been implicated in several stages of membrane trafficking, but the role of lipids in vesicle budding and fusion in living cells is poorly understood. It is known that the sphingolipid synthesis pathway is necessary for trafficking of glycosylphosphatidylinositol (GPI)-anchored proteins from the endoplasmic reticulum to the Golgi apparatus (Horvath *et al*., 1994; Sütterlin *et al*., 1997). Phospholipids, in particular phosphorylated derivatives of phosphatidylinositol (PtdIns), also appear to play a critical role in regulating transport events (De Camilli *et al*., 1996). Sterols have been implicated in control of the internalization step of endocytosis in both yeast and mammalian cells (Munn *et al*., 1999; Rodal *et al*., 1999; Subtil *et al*., 1999). Sphingoid bases have also been shown to play a role in regulation of cell surface expression of amino acid permeases (Skrzypek *et al*., 1998).

Using the *S. cerevisiae lcb1-100* mutant, a requirement for sphingoid base synthesis for the internalization step of endocytosis and for proper actin cytoskeleton organization was revealed (Zanolari *et al*., 2000). The *LCB1* gene encodes a subunit of the serine palmitoyltransferase that catalyses the first step in sphingolipid synthesis, the condensation of serine and palmitoyl-CoA to yield 3-ketosphinganine (Nagiec *et al*., 1994). The sphingoid base requirement for endocytosis can be suppressed by loss of protein phosphatase 2A activity or by overexpression of two kinases (Pkc1p or Yck2p), suggesting a signaling function of sphingoid bases in activation of a protein kinase and a protein phosphatase acting sequentially in endocytosis (Friant *et al*., 2000).

These results imply that the function of sphingoid bases in endocytosis is to control protein phosphorylation. In mammalian cells, studies revealed that sphingosine induces *in vitro* phosphorylation of endogenous proteins through the activation of protein kinases (Pushkareva *et al*., 1992) and two unidentified sphingosine-activated protein kinases were characterized by their substrate specificity and their sphingosine requirement (Pushkareva *et al*., 1993). Sphingosine acts also by inhibiting some kinases like protein kinase C (Hannun *et al*., 1986) or by activating some others, like the casein kinase II (McDonald *et al*., 1991), the atypical protein kinase C isoform ζ (Muller *et al*., 1995), the p21 activated kinase-1 (PAK1; Bokoch *et al*., 1998) and the 3-phosphoinositide-dependent kinase-1 (PDK1; King *et al*., 2000). Thus sphingoid bases play a dual role in the regulation of protein phosphorylation.

It was recently shown that sphingosine stimulates PDK1 kinase autophosphorylation and increases the phosphorylation of known PDK1 substrates like PAK1, Akt and PKC β kinases *in vitro* suggesting that sphingosine is a novel activator of PDK1

(King *et al*., 2000). PDK1 phosphorylates several protein kinases *in vitro* and is responsible for activating these enzymes *in vivo* (Alessi *et al*., 1998; Le Good *et al*., 1998; Pullen *et al*., 1998). *S. cerevisiae* has two PDK1 homologs encoded by the *PKH1* and *PKH2* genes. Similarly, Pkh1/2p kinases were shown to phosphorylate and activate several protein kinases including Ypk1/2p and Pkc1p both *in vitro* and *in vivo* (Casamayor *et al*., 1999; Inagaki *et al*., 1999). Pkh1p and Pkh2p kinases share an essential role for cell growth since the double knockout *pkh1Δ pkh2Δ* yeast strain is not viable. Synthetic lethality of *pkh1Δ pkh2Δ* double mutant is complemented by full length human PDK1, or human PDK1 lacking the C-terminal pleckstrin homology (PH) domain that was shown to bind phosphoinositides (Casamayor *et al*., 1999). Furthermore both Pkh1p and Pkh2p kinases contain no obvious PH domain, in contrast to Human and Drosophila PDK1, suggesting that in yeast the activation of Pkh1/2p kinases may not be dependent on phosphoinositides.

A recent study revealed that yeast cells overproducing *PKH1* display an increased resistance to myriocin treatment, a serine palmitoyl transferase (Lcb1p) inhibitor (Sun *et al*., 2000). Pkh2p was shown to phosphorylate Pkc1p kinase *in vitro* and the temperature-sensitivity of a *pkh-ts* (*pkh1-ts pkh2Δ*) mutant is partially suppressed by a *PKC1-R398P* dominant mutation (Inagaki *et al*., 1999). In a previous study, we showed that *PKC1* overexpression or *PKC1-R398P* expression can suppress the sphingoid base requirement for endocytosis, suggesting a link between sphingoid base synthesis and Pkc1p activation (Friant *et al*., 2000). These results suggest that the Pkh1/2p kinases may be activated by sphingoid bases and play a role in endocytosis via Pkc1p activation. To test this hypothesis, we investigated the implication of Pkh1/2p kinases in endocytosis.

Here, we show that overexpression of one of two protein kinases, Pkh1p or Pkh2p, can abrogate the sphingoid base synthesis requirement for endocytosis and restore a proper organization of the actin cytoskeleton in *lcb1-100* mutant cells. The two kinases have an overlapping function in endocytosis because only a mutant with impaired function of both kinases is defective in the internalization step of endocytosis. Furthermore, phosphorylation of Pkc1p by Pkh1p or Pkh2p kinase *in vitro* is increased in the presence of nanomolar concentration of sphingoid base. These results imply that the function of sphingoid base in endocytosis is to activate a signaling pathway via Pkh1/2p kinases. The ultimate target of the sphingoid base-mediated signaling pathway may be the endocytic machinery via a novel effector, the amphiphysin-homolog Rvs167p (R. Lombardi *et al*., accompanying study) and/or the actin cytoskeleton via the Pkc1p kinase.

Results

Overexpression of Pkh1/2p kinases restores endocytosis in the *lcb1-100* mutant

To test if yeast PDK1 homologs, Pkh1/2p kinases, could be part of the sphingoid base requirement for endocytosis, we analyzed if *PKH1* or *PKH2* overexpression could suppress the endocytic defect of the *lcb1-100* mutant. The *lcb1-100* strain was transformed with high copy (*2μ*) plasmids bearing *PKH1* or *PKH2* genes (Table I). Neither of the two protein kinases tested was able to suppress the temperature-sensitive growth phenotype displayed by the *lcb1-100* mutant (data not shown). The same strains overexpressing Pkh1p or Pkh2p kinases were tested for internalization of [^{35}S]α-factor at 37°C. High copy expression of PKH2 and to a less extent PKH1 restored the defect in

α-factor internalization of the *lcb1-100* mutant cells at 37°C (Figure 1A). To compare the suppressor effect of the Pkh1/2p kinases, we tested in parallel the two other kinases, Yck2p and Pkc1p, that were already shown to restore the *lcb1-100* defect in endocytosis (Figure 1A; Friant *et al*., 2000). The α-factor uptake rate displayed by the *lcb1-100* cells overexpressing *PKH2* overlaps more or less with the one overexpressing *YCK2*, whereas *PKH1* suppressor effect is more similar to the one of *PKC1* (Figure 1A).

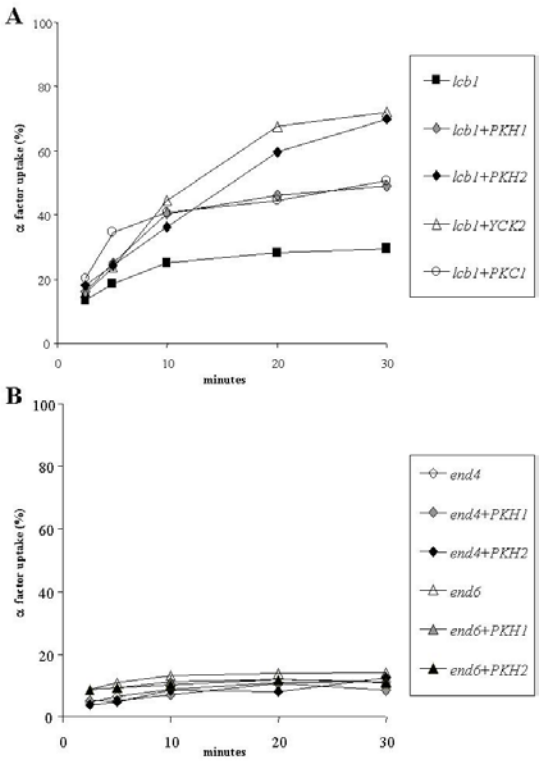

Figure 1. Overexpression of *PKH1* and *PKH2* specifically suppresses the *lcb1-100* α-factor internalization defect
(A) Strain RH3802 (*lcb1*) was transformed with high copy plasmids carrying *PKH1*, *PKH2*, *PKC1* or *YCK2* kinase genes (Table I) and the corresponding transformants were assayed for α-factor internalization at 37°C and compared with *lcb1-100* cells. (B) The *sla2-41* (*end4-1*) and *end6-1* (*rvs161*) temperature sensitive mutants (RH3802, RH1597 and RH2082) were transformed with a high copy number plasmid bearing *PKH1* or *PKH2* and assayed for α-factor uptake at 37°C.

To determine if other endocytosis mutants could also be suppressed by *PKH1* or *PKH2* overexpression, the *sla2-41* (*end4-1*) and *end6-1* (*rvs161*) strains were transformed with the *2μ* plasmids bearing the *PKH1/2* genes and assayed for α-factor uptake at 37°C (Figure 1B). These two mutants are defective for α-factor internalization at 37°C (Munn *et al*., 1995; Raths *et al*., 1993). The high copy expression of *PKH1* or *PKH2* did not restore endocytosis in these strains (Figure 1B), showing that Pkh1/2p over-expression specifically suppresses the *lcb1-100* endocytic defect.

The ability of *lcb1-100* cells overexpressing *PKH1* or *PKH2* to carry out fluid-phase endocytosis at 37°C was also tested and compared to wild type and *lcb1-100* cells (Figure 2). The *lcb1-100* mutant showed a few small vacuoles when viewed by Nomarski optics and was defective for uptake and accumulation of the fluorescent dye lucifer yellow (LY) in the vacuole at 37°C (Zanolari *et al*., 2000). Consistent with the α-factor uptake results, overexpression of *PKH1* or *PKH2* allowed the *lcb1-100* mutant to accumulate LY in the vacuole even tough the vacuole morphology defect was not restored (Figure 2).

Taken together, these results show that overexpression of Pkh1/2p kinases specifically suppress both receptor-mediated and fluid-phase endocytosis defects of the *lcb1-100* mutant. Furthermore, they support the notion that sphingoid bases may activate a protein phosphorylation pathway required for the internalization step of endocytosis and mediated via Pkh1/2p kinases.

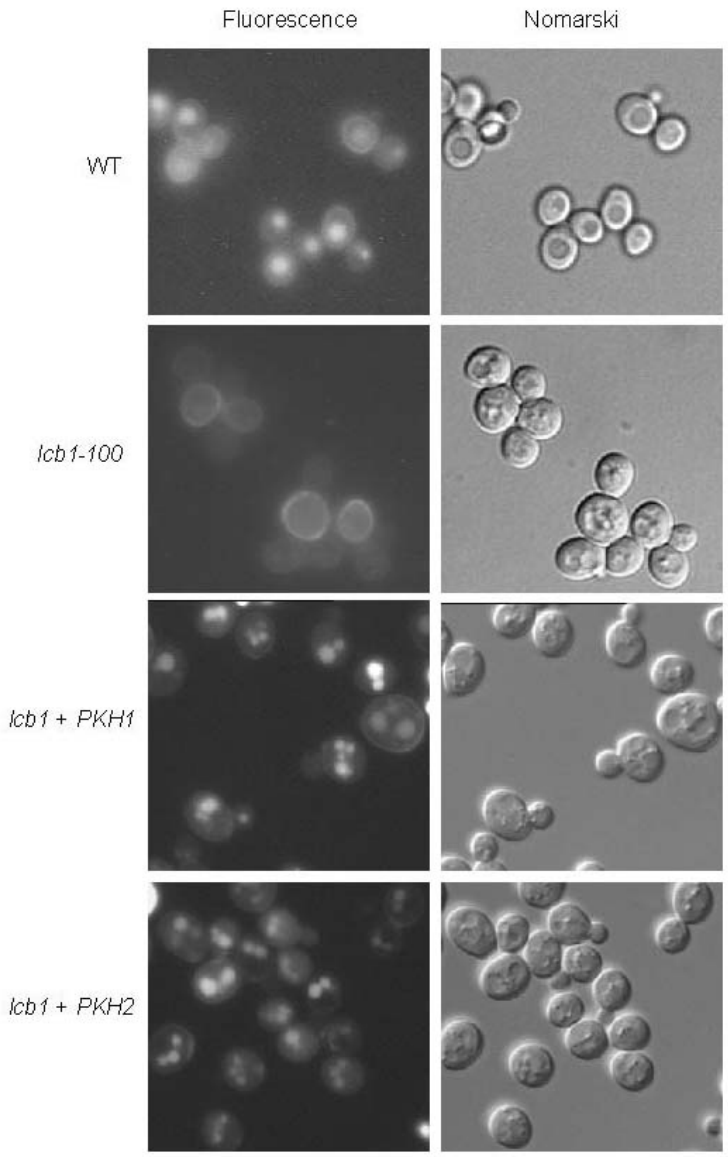

Figure 2. *PKH1/2* overexpression suppresses the fluid-phase endocytic defect of the *lcb1-100* mutant
Wild-type cells (WT, RH448) and *lcb1-100* (*lcb1*, RH3802) cells carrying either a *PKH1* or a *PKH2* high copy number plasmid (*lcb1+PKH1* or *lcb1+PKH2*) were assayed for LY accumulation in the vacuole at 37°C. The same field of cells viewed by fluorescence (left panel) and by Nomarski optics (right panel) is shown. Note that *lcb1-100* cells have fragmented vacuoles when compared to wild-type cells (right panel).

Pkh1/2p kinases are required for endocytosis

To determine whether Pkh1/2p kinase activity is required for the internalization step of endocytosis, α-factor uptake and LY accumulation of *pkh1Δ*, *pkh2Δ* and *pkh-ts* strains were assayed (Figure 3 and 4). *PKH1* and *PKH2* have overlapping function for cell growth (Casamayor *et al*., 1999; Inagaki *et al*., 1999). The *pkh-ts* strain harbors a chromosomal deletion of the *PKH2* gene (*pkh2::LEU2*) and a temperature-sensitive *pkh1-ts* mutant allele ($pkh1^{D398G}$; Inagaki *et al*., 1999). The single disruptants and temperature-sensitive mutant strains were assayed for α-factor uptake and compared to wild-type cells (Figure 3A and 3B). The single mutant cells *pkh1Δ* and *pkh2Δ* internalized

α-factor with the same rate as wild-type cells at 37°C (Figure 3A). At 24°C, the *pkh-ts* strain showed a partial α-factor internalization defect when compared to wild-type cells, and at 37°C the mutant strain was unable to internalize α-factor (Figure 3B). Therefore, we conclude that Pkh1p and Pkh2p kinases have an overlapping function for endocytosis and that the loss of Pkh1/2p kinase activity affects the internalization step of receptor-mediated endocytosis.

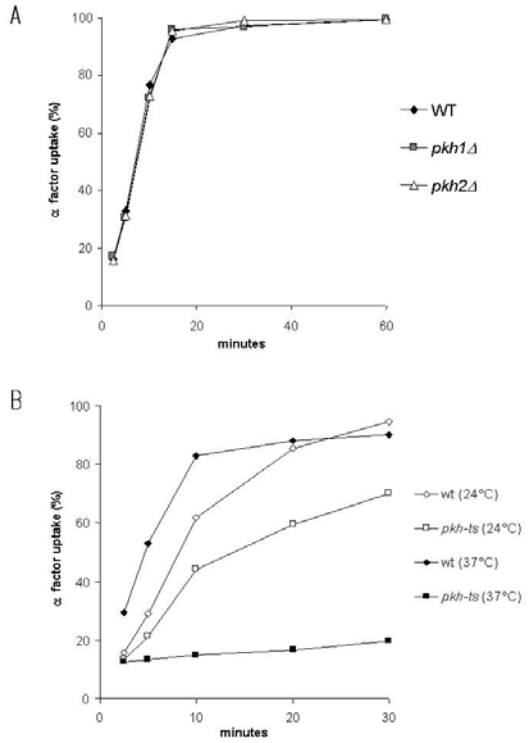

Figure 3. Pkh1/2p are required for receptor-mediated endocytosis
(A) Pkh1p and Pkh2p have redundant function in endocytosis. The single mutant *pkh1Δ* (RH3802) and *pkh2Δ* (RH4336) cells were assayed for α-factor uptake at 37°C and compared to the wild-type cells (WT, RH448). (B) Pkh1/2p kinases are required for endocytosis. Radiolabeled α-factor uptake assays were performed at 24°C (open symbols) or 37°C (closed symbols) on wild-type (RH448) and *pkh-ts* (RH4329) strains.

As the *pkh-ts* mutant is defective for receptor-mediated internalization at 37°C it was important to examine whether this mutant is also defective for fluid-phase endocytosis. Therefore, we tested for a defect using the LY uptake assay. The *pkh-ts* cells were completely defective in LY accumulation at 37°C (Figure 4). These results show that Pkh1p and Pkh2p have an overlapping function in endocytosis, because only the cells with impaired activity for both kinases were defective for the internalization step of endocytosis.

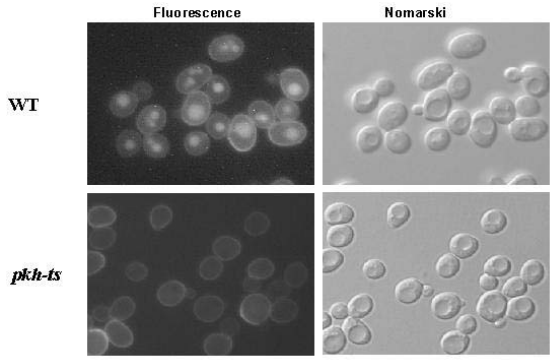

Figure 4. Pkh1/2p kinases are required for fluid-phase endocytosis
Wild-type (WT, RH448) and *pkh-ts* (RH4598) cells were incubated with LY for 1 hr at 37°C. To visualize LY uptake, cells were viewed by FITC-fluorescence optics (left panel). The same fields of cells were viewed by Nomarski optics (right panel) to visualize the vacuoles.

Overexpression of Pkh1/2p specifically corrects the actin defect of the *lcb1-100* mutant

The *lcb1-100* mutant is defective in the organization of the actin cytoskeleton at 37°C. This defect, like the endocytic defect, can be suppressed by addition of phytosphingosine (PHS) or overexpression of Yck2p or Pkc1p kinases in the *lcb1-100* cells (Friant *et al*., 2000; Zanolari *et al*., 2000). Therefore, it is conceivable that overexpression of the Pkh1/2p kinases, which restored endocytosis in the *lcb1-100* mutant, may also correct the actin organization defect of this mutant. To test this, we examined whether Pkh1p or Pkh2p overexpression in the *lcb1-100* mutant could restore the polarized distribution of actin at 37°C. Wild-type, *lcb1-100* and

lcb1-100 overexpressing *PKH1* or *PKH2* cells were grown at 24°C, shifted to 37°C for 2 hours and the cells were fixed and stained with TRITC-phalloidin to visualize F-actin (Figure 5). A shift from 24°C to 37°C causes a heat-induced reorganization of the actin cytoskeleton in wild-type yeast cells to a non-polarized distribution. Normal polarized actin localization is restored after 1.5-2 h at 37°C in wild type cells (Figure 5). In contrast, this perturbation was irreversible in the *lcb1-100* mutant cells, as seen by the accumulation of actin patches in the mother cell of the budded cells (Figure 5; Zanolari *et al*., 2000). However, *lcb1-100* mutant cells that were suppressed for endocytosis either by Pkh1p or Pkh2p kinase overexpression, displayed polarized distribution of actin that was more similar to wild-type cells with cortical actin patches concentrated in the bud (Figure 5).

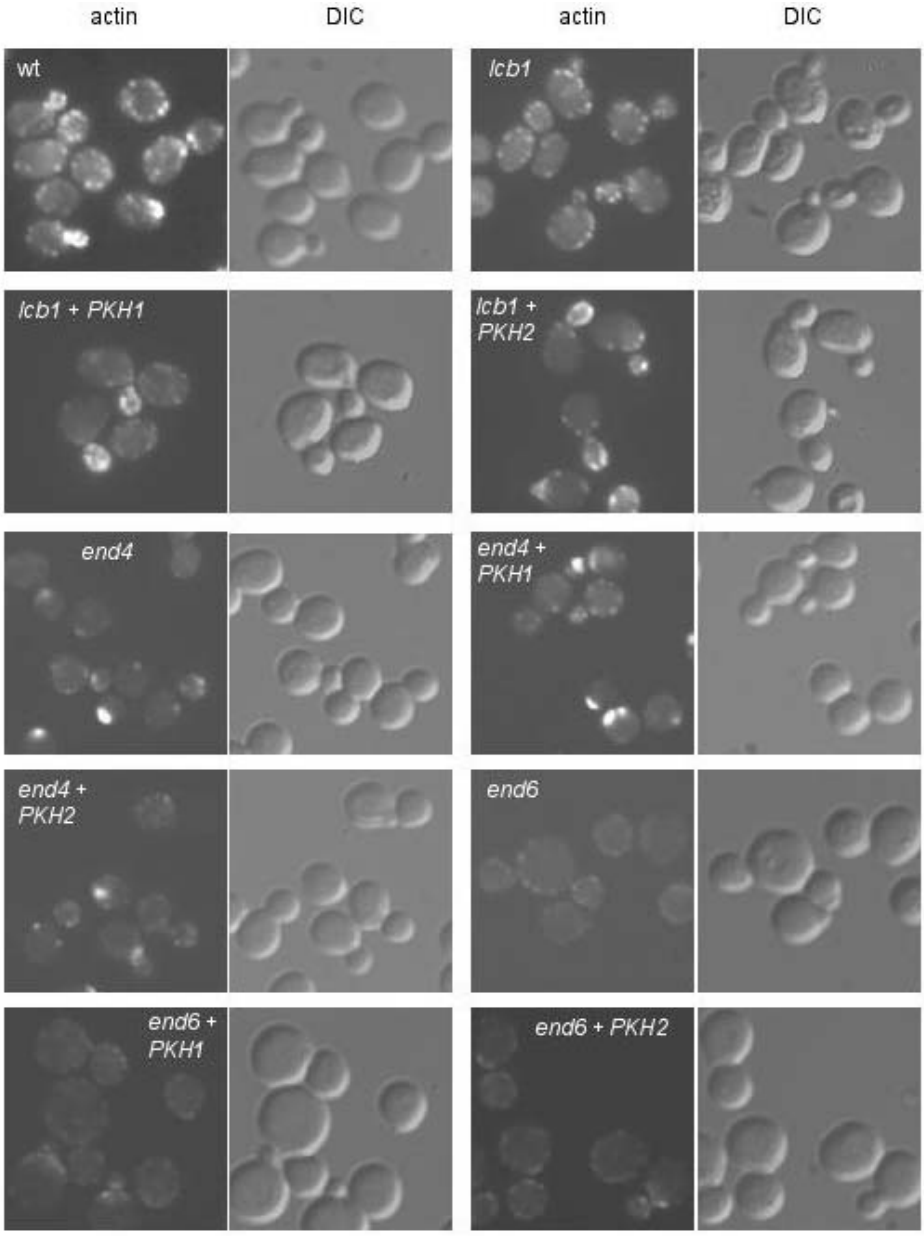

Figure 5. Pkh1/2p kinases overexpression suppresses specifically the *lcb1-100* actin organization defect
Logarithmic cultures of wild-type cells (WT, RH448), *lcb1-100*, *end4-1* (*sla2-41*) and *end6-1* (*rvs161*) mutant cells (RH3802, RH1597 and RH2082) without or with the *PKH1* and *PKH2* plasmids, were grown at 24°C, shifted to 37°C for 2 hr, fixed, stained with TRITC-phalloidin and observed by fluorescence (actin) and Nomarski (DIC) microscopy.

Pkh1/2p kinases have been shown to be required for proper actin cytoskeleton organization in yeast (Inagaki *et al*., 1999). To determine if the suppression by *PKH1/2* was specific for the sphingoid base synthesis requirement of *lcb1-100* cells, two other endocytic mutant that display actin cytoskeleton defects the *sla2-41* (*end4-1*) and *end6-1* (*rvs161*), were tested (Munn *et al*., 1995; Raths *et al*., 1993). The *sla2-41* and *end6-1* mutants were transformed with the high copy number plasmids bearing *PKH1* or *PKH2* genes and assayed for actin localization at 37°C (Figure 5). Proper actin localization was not restored by either *PKH1* or *PKH2* overexpression in these mutants, showing that suppression is specific for the *lcb1-100* mutant. The above results suggest that one target of the sphingoid base synthesis requirement is likely to be the actin cytoskeleton because overexpression of the Pkh1/2p kinases specifically corrected the actin defect in the *lcb1-100* mutant.

Sphingoid base activates Pkh1p and Pkh2p kinase activity *in vitro*

We could show that Pkh1p and Pkh2p kinases are required for endocytosis and that their overexpression can suppress the requirement for sphingoid base synthesis. The above results suggest that like their mammalian homologue PDK1, Pkh1p and Pkh2p could be directly activated by sphingoid base as part of a signaling cascade required for the internalization step of endocytosis. To test this hypothesis, we developed an *in vitro* phosphorylation assay by using immunoprecipitated Pkh1p or Pkh2p incubated in presence of increasing concentration of phytosphingosine (PHS). Wild-type cells were transformed with a plasmid bearing *PKH1* or *PKH2* genes tagged at their C-terminus with a triple HA epitope (Table I). These two Pkh1-HA_3 and Pkh2-HA_3 constructs are functional because they can suppress the temperature sensitive growth phenotype displayed by the *pkh-ts* strain (data not shown). It was recently shown that Pkh1/2p kinases directly phosphorylate and thus activate Pkc1p (Inagaki *et al*., 1999). Therefore, we used immunoprecipitated Pkc1p as the natural substrate for Pkh1p and Pkh2p kinase in our *in vitro* assay. A wild-type strain was transformed with a multicopy plasmid encoding Pkc1p triple HA tagged at its C-terminus (pTS94). This construct is functional since a *pkc1-2* strain transformed with this plasmid shows no growth defect at 37°C anymore. Pkh1-HA_3, Pkh2-HA_3 or Pkc1-HA_3 were immunoprecipitated with an anti-HA antibody. Immunoprecipitated Pkc1-HA_3 shows a proper folding and activity because in an *in vitro* phosphorylation assay the immunoprecipitated Pkc1-HA_3 phosphorylated correctly its substrate, the myelin basic protein (Antonsson *et al*., 1994; data not shown). Immunoprecipitated Pkh1-HA_3 or Pkh2-HA_3 was incubated in presence of its substrate Pkc1-HA_3, and aliquots of this kinase-substrate mix were incubated with [γ-^{32}P]ATP in the presence of increasing concentration of phytosphingosine (PHS) (Figure 6). A control without Pkh1/2p kinase to determine the amount of Pkc1-HA_3 autophosphorylation was done in parallel. Phosphorylated [^{32}P]Pkc1-HA_3 was revealed by using a Cyclone Storage Phosphor Imager (Packard) after electrophoresis on a 7.5% SDS-PAGE (Figure 6A and 6C) and the amount of radiolabeled Pkc1-HA_3 was quantified (Figure 6B and 6D). All phosphorylation assays were performed at least twice, the results shown are from one of the independent experiments that gave nearly identical results. PHS was found to be a potent activator of both Pkh1p and Pkh2p kinases, since at concentration as low as 0.5 nM there is an increase in Pkc1-HA_3 phosphorylation (Figure 6A and 6C)

therefore an activation of Pkh1/2p kinase activity. A 3-fold activation of both Pkh1p and Pkh2p kinase activity is observed in the presence of 2.5 nM to 25 nM PHS respectively (Figure 6B and 6D). However, PHS becomes less effective at higher concentrations and at 500 nM PHS inhibited both Pkh1p and Pkh2p phosphorylation of Pkc1p (Figure 6). These findings suggest that PHS has a bifunctional effect on Pkh1/2p activity *in vitro*, stimulating the activity at low concentrations and inhibiting it at high concentrations. These results show that Pkh1/2p kinases are directly activated by PHS *in vitro* and are one of the yeast sphingoid base-activated kinases.

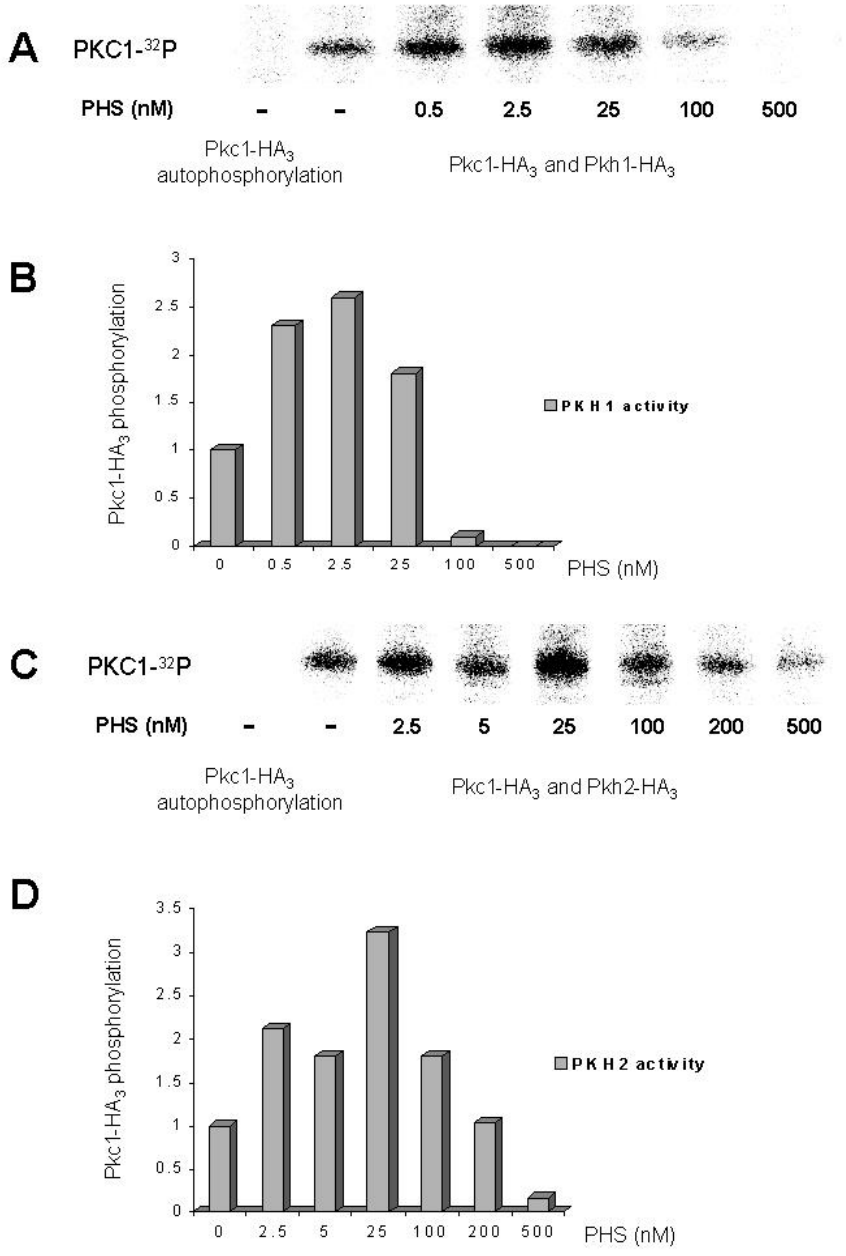

Figure 6. Phytosphingosine activates the Pkh1/2p kinases
(A) Immunoprecipitated Pkh1-HA_3 was incubated with its substrate Pkc1-HA_3 in the presence of [γ-^{32}P]ATP and increasing amounts of PHS. Phosphorylated Pkc1-HA_3 was revealed by a Cyclone Storage Phosphor Imager (Packard) after separation on a 7.5% SDS-PAGE. (B) The amount of radiolabeled [^{32}P]Pkc1-HA_3 was quantified in each lane and basal phosphorylation of Pkc1p by Pkh1p without PHS was set as 1. (C) and (D) The same assay was done using Pkh2-HA_3 as the kinase.

Discussion

The major finding of this study is that Pkh1/2p kinases are part of a sphingoid base-mediated signaling pathway required for the internalization step of endocytosis and for proper actin cytoskeleton organization in yeast. As shown previously, the actin cytoskeleton plays an essential role in the internalization step of endocytosis in yeast, because yeast mutants in actin and actin-binding proteins are defective in endocytosis (Kübler and Riezman, 1993; Munn *et al*., 1995). Here we showed that overexpression of *PKH1/2* restored the endocytic and the actin cytoskeleton organization defect of the *lcb1-100* mutant. It is interesting to note, that neither *PKH1* nor *PKH2* over-expression restored the endocytic or actin defect of other mutants that are defective in the internalization step of endocytosis and in actin cytoskeleton organization, meaning that this suppressor effect was specific for the *lcb1-100* mutation. We show that Pkh1p and Pkh2p kinases have redundant functions for both fluid-phase and receptor-mediated endo-cytosis. A previous study has shown that these two kinases are also required for actin cytoskeleton organi-zation, since the *pkh-ts* strain displays a defect in actin polarization upon shift to 37°C (Inagaki *et al*., 1999). These results suggest that Pkh1/2 kinases play adirect role in endocytosis perhaps by regulating the endocytic machinery and/or the actin cytoskeleton. Here, we showed that sphingoid base activates both Pkh1p and Pkh2p protein kinases *in vitro*. All these results together suggest that there is a sphingoid base-mediated signaling path-way required for endocytosis and that the sphingoid base activated kinase that mediates this cascade are the Pkh1/2p kinases.

Pkh1p and Pkh2p are the yeast homologs of mammalian PDK1 kinase. Human, Drosophila and *C. elegans* PDK1 kinases contain a pleckstrin homology (PH) domain and this PH domain binds $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Currie *et al*., 1999; Fruman *et al*., 1999; Stephens *et al*., 1998). In contrast, the two yeast members of this kinase family, Pkh1p and Pkh2p, lack the PH domain (Casamayor *et al*., 1999). It was previously shown that mammalian PDK1 lacking the PH domain was sufficient to rescue *pkh1Δ pkh2Δ* mutant cells from lethality (Casamayor *et al*., 1999). Furthermore, yeast cells are not able to generate $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Hawkins *et al*., 1993) and Pkh1p does not bind $PI(3,4,5)P_3$ under conditions where this phospholipid binds tightly to PDK1 (R. Currie and C.P. Downes, unpublished data). These findings suggest that Pkh1/2 kinases are not regulated by phospho-inositides like their mammalian counterpart. The best candidates for regulating Pkh1/2p kinases activity are sphingoid bases, because a previous study showed that mammalian PDK1 kinase is activated *in vitro* in presence of sphingosine and that elevated intracellular sphingosine level increases PDK1 activity *in vivo* (King *et al*., 2000). Here, we showed that overexpression of *PKH1* or *PKH2* suppresses the endocytic defect of the *lcb1-100* mutant strain that is impaired in sphingoid base synthesis. Moreover, by using an *in vitro* kinase assay we could show that both Pkh1p and Pkh2p are activated by nanomolar concentration of phytosphingosine (PHS). There is a three fold increase in Pkh1/2p kinases activity in presence of very low concentrations of PHS (0.5 to 2.5 nM). Using the same *in vitro* kinase assay, we also tested the ability of dihydrosphingosine (DHS), another sphingoid base that was shown to suppress the *lcb1-100* endocytic defect (Zanolari *et al*., 2000), to activate Pkh1p kinase. We obtained the same results as for PHS (data not shown). All these results together suggest that both PDK1 and its yeast counterparts Pkh1/2p kinases are sphingoid base-activated kinases. Further-more, the results obtained here together with previous results showing that neither

phosphorylated sphingoid bases nor ceramides or sphingolipids are required for endocytosis (Zanolari *et al*., 2000), suggest that sphingoid bases (DHS and/or PHS) are required for Pkh1/2 kinases activation. However, we cannot rule out the possibility that another yet unidentified derivative of PHS/DHS could be the true Pkh1/2 kinase activator, but this derivative cannot be phosphorylated sphingoid base because yeast incapable of generating phosphorylated sphingoid bases are capable of endocytosis (Zanolari *et al*., 2000).

Pkc1p and Ypk1/2p kinases were identified as downstream effectors of the Pkh1/2p protein kinase cascade. These two kinases are directly phosphorylated by Pkh kinase *in vitro* (Casamayor *et al*., 1999; Inagaki *et al*., 1999). Reduced Pkc1p activity was observed in a *pkh-ts* mutant strain indicating that Pkh1/2p kinases are required for Pkc1p function *in vivo* (Inagaki *et al*., 1999). In a previous study, we could show that an increased gene dosage of *PKC1* or expression of a dominant, activated allele of *PKC1* suppresses the endocytic defect of the *lcb1-100* mutant (Friant *et al*., 2000). The above results showed that Pkc1p phosphorylation by Pkh1/2p kinases is activated by sphingoid base *in vitro*. These data suggest that Pkc1p may be activated by Pkh1/2p kinases in response to sphingoid base and that this signaling cascade is required for the internalization step of endocytosis. The essential role for Pkh1/2 kinases in endocytosis is further confirmed by the strong endocytic defect observed in a *pkh-ts* mutant strain. The *lcb1-100* mutant strain blocked in sphingoid base synthesis shows a similar endocytosis defect (Zanolari *et al*., 2000), whereas the *pkc1-ts* mutant cells showed wild-type endocytosis (Friant *et al*., 2000). Therefore, the Pkc1p kinase is one of the downstream effectors of the Pkh1/2p signaling cascade, but it is not the only one required for the internalization step of endocytosis. Yck2p kinase could be another downstream effector, because overexpression also suppresses the *lcb1-100* endocytosis defect and only the double mutant *pkc1-ts yck-ts* strain is completely defective for endocytosis (Friant *et al*., 2000). We did not find a consensus PDK1/2-Pkh1/2 phosphorylation site in the Yck2p kinase, whereas both Pkc1p and Ypk1/2p kinases have these site (Casamayor *et al*., 1999), meaning that Yck2p is probably not directly activated by Pkh1/2p. There could be another kinase upstream of Yck2p in the signaling cascade. The Ypk1/2p kinases would be good candidates.

The Ypk1p kinase was recently identified as a multicopy suppressor gene that restores growth in presence of a serine palmitoyl transferase (Lcb1p) inhibitor. Furthermore, phosphorylation of the Ypk1p kinase is increased in presence of PHS (Sun *et al*., 2000). This phosphorylation could be due to activation of Pkh1/2p kinases by PHS, because Ypk1p is directly phosphorylated by Pkh2p *in vitro* (Casamayor *et al*., 1999). In agreement with this hypothesis, it was shown that Pkh kinase activity was required for maximal Ypk1p phosphorylation *in vivo* (Casamayor *et al*., 1999). These data suggest that Pkh1/2p kinases might be tightly regulated by sphingoid bases, not only for their function in endocytosis, but for their overall function in regulating several yeast processes, like cell growth, control of the mitogen-activated protein kinases and organization of the actin cytoskeleton. In a previous study, we could show that *YPK1* or *YPK2* overexpression did not suppress the *lcb1-100* endocytosis defect (Friant *et al*., 2000), suggesting that these kinases are not part of the signaling cascade required for the internalization step of endocytosis. However, we cannot exclude that *YPK1/2* overexpression would not be sufficient to mimic the Pkh1/2p activation effect on these kinases, because they may not be active in their dephosphorylated state, even if overexpressed.

In summary, our results are the first example of a sphingoid base activated signaling pathway being used to regulate a step of membrane traffic. Many details remain to be discovered, including the identification of the downstream effectors of the Pkh1/2p sphingoid base activated protein kinase and the mechanism whereby sphingoid base controls the relative activities of protein kinases. The ultimate target of the sphingoid base mediated signaling pathway may be the endocytic machinery via a novel effector, the amphiphysin homolog Rvs167p (R. Lombardi *et al*., accompanying study; Lombardi and Riezman, 2001) and/or the actin cytoskeleton via the Pkc1p kinase. The ease of genetic and molecular studies in yeast should help to understand these questions.

Materials and Methods

Plasmids, strains, media and genetic manipulations

Plasmids and yeast strains used in this study are listed in Tables I and II, respectively. Disruption mutants were created by integrative transformation using standard techniques. Yeast cell cultures and genetic manipulations were carried out essentially as described (Sherman *et al*., 1983). Yeast cells were transformed by the LiAc method using single-stranded carrier DNA and DMSO (Hill *et al*., 1991; Schiestl and Gietz, 1989). Rich YPUAD medium and synthetic minimal media (SD) complemented with the appropriate nutrients for plasmid maintenance were prepared as described (Munn *et al*., 1995).

Endocytosis assays

Lucifer yellow-carbohydrazide (LY) (Fluka, Buchs, Switzerland) assays were performed as described (Dulic *et al*., 1991; Munn and Riezman, 1994). Yeast precultures were grown at 24°C in SD selective media in order to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD to mid log phase, shifted to 37°C for 15 min and incubated for 1 h at 37°C with LY. [^{35}S]α-factor uptake assays were performed on mid-log phase cells using the continuous presence protocol as described (Dulic *et al*., 1991). Pre-cultures were done at 24°C in SD selective media in order to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD medium, the α-factor uptake assays were carried out at 24°C or 37°C after 15 min preincubation at the respective temperature. All uptake assays were performed at least twice, the results shown are from one of the independent experiments, which gave nearly identical results.

Rhodamine-Phalloidin staining of actin

Yeast cells pre-cultures were grown at 24°C in SD selective media in order to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD medium to early log phase. Cells at 1 x 10^7 cells/ml were then incubated for 2 hr at 37°C, fixed in formaldehyde and stained with TRITC-phalloidin (Sigma, St. Louis, MO) to visualize F-actin essentially as described previously (Benedetti *et al*., 1994).

Table I. Plasmids

Plasmid	Yeast Ori	Insert	Source
YEp195-PKH1	*2μ*	*PKH1*	Schmelzle and Hall
YEp195-PKH2	*2μ*	*PKH2*	Schmelzle and Hall
pTS80	*2μ*	*PKH1-HA$_3$*	Schmelzle and Hall
pTS81	*2μ*	*PKH2-HA$_3$*	Schmelzle and Hall
pSH24	*2μ*	*PKC1*	Helliwell *et al*., 1998
pTS101	*2μ*	*PKC1-HA$_3$*	Schmelzle and Hall
pL2.3	*2μ*	*YCK2*	Robinson *et al*., 1992

Table II. Yeast strains

Strains	Genotype	Source
RH448	*leu2 ura3 his4 lys2 bar1*	Lab. Strain
RH1597	*leu2 ura3 his4 bar1 end4-1*	Raths *et al*., 1993
RH2082	*leu2 ura3 his4 bar1 end6-1*	Munn *et al*., 1995
RH3802	*leu2 ura3 his4 his3 ade2 lys2 bar1 lcb1-100*	Friant *et al*., 2000
RH3809	*leu2 ura3 his4 bar1 lcb1-100*	Friant *et al*., 2000
RH5388	*leu2 ura3 ade2 trp1 bar1::URA3 pkh2::LEU2*	This study
RH5411	*leu2 ura3 his2 ade1 trp1 bar1::URA3*	This study
RH5412	*leu2 ura3 his2 ade1 trp1 bar1::URA3 pkh1-ts pkh2::LEU2*	This study
RH5413	*leu2 ura3 his3 ade2 trp1 lys2 bar1 pkh1::TRP1*	This study

All strains listed in this table are *MAT* **a**.

In vitro phosphorylation assay

Wild-type yeast cells (RH448) transformed with plasmids bearing *PKC1-HA$_3$*, *PKH1-HA$_3$* or *PKH2-HA$_3$* were grown at 24°C in SD selective media in order to maintain the plasmids. Cells around 1.5 x 10^7 cells/ml were then harvested and total yeast proteins were extracted by glass beads lysis in a buffer containing, 50 mM HEPES pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol and 1 μg/ml of the protease inhibitors aprotinin, leupeptin, pepstatin A, chymostatin and antipain. The protein concentration of the lysates was measured using the Bio-Rad protein assay. Equal amounts of total protein for the different kinases were taken and triple HA-tagged kinases were immunoprecipitated over-night at 4°C from these extracts using 5 μl of anti-HA rat monoclonal antibody (Clone 3F10, Boehringer Manheim), 50 μl of 30% protein G-sepharose beads in a final volume of 750 μl immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40 and 1 μg/ml of the protease inhibitors aprotinin, leupeptin, pepstatin A, chymostatin and antipain). Beads were then washed three times in immuno-precipitation buffer and two times in 40 mM MOPS pH 7.5 buffer and resuspended in phosphorylation buffer (40 mM MOPS pH 7.5, 1 mM DTT and 10 mM $MgCl_2$). Equal amounts of kinase-beads (Pkh1p or Pkh2p beads) were mixed with Pkc1p-beads (substrate). In order to determine the amount of autophospho-rylation, a control containing the same amount of Pkc1p beads mixed with phosphorylation buffer was treated in parallel. The kinase-substrat mix was aliquoted and 1 μl of D-erythro-phyto-sphingosine (PHS) at the proper dilution in ethanol was added to the mix in order to get a final concentration of 0.5, 2.5, 25, 100, 200 or 500 nM of PHS in each reaction assay. The control reaction without PHS was done by adding 1 μl of ethanol to the reaction. After 10 min of pre-incubation at room temperature, 2 μl of

[^{32}P]ATP mix (1 mM ATP and 4 μCi [γ-^{32}P]ATP) was added and the phosphorylation reaction was run for 30 min at room temperature. The reaction was stopped by addition of cold ATP at a final concentration of 50 mM and Laemmli protein sample buffer. Samples were boiled for 5 min and the total reaction was loaded on a 7.5 % SDS-PAGE. After electrophoresis, the gel was washed once in 12.5% TCA and two times in Coomassie blue destaining solution. Phosphorylated Pkc1-HA_3 was revealed and quantified by using a Cyclone Storage Phosphor Imager (Packard). All phosphorylation assays were performed at least twice. The results shown are from one of the independent experiments, which gave nearly identical results.

Acknowledgments

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Regulation of the endocytic function of Rvs167p-complex by phosphorylation

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Abstract:

The internalization step of endocytosis in yeast requires the actin cytoskeleton and a large number of actin-associated proteins. To understand the functions of the different proteins involved in this step we have looked for protein-protein interactions among them. We find that three proteins involved in this process, Rvs167p, Myo5p and Sla2p/End4p, interact with each other and form a 600 kDa protein complex most likely involved in regulating the actin cytoskeleton. We also identified Rvs167p as an important target for regulation of endocytosis by Pkh2p protein kinase. Pkh1/2p kinases are homologous to mammalian 3-phosphoinositide-dependent kinase-1 (PDK1). By sequence comparison we identified a putative phosphorylation site at the extreme N-terminus of Rvs167p and we could show that Pkh2p is able to phosphorylate Rvs167p *in vitro*. We mutated the phosphorylated Thr7 to an alanine and demonstrate that this point mutation affects the internalization step of endocytosis *in vivo*. Interestingly, the point mutation did not affect the actin cytoskeleton suggesting a possible direct function of Rvs167p in endocytosis besides its role in actin cytoskeleton function.

I wrote the manuscript and performed all shown experiments.

Introduction

Endocytosis is a general mechanism whereby eukaryotic cells internalize portions of their own plasma membrane together with molecules from the external environment. Several studies in the yeast *Saccharomyces cerevisiae* have identified a large number of mutants defective in the internalization step of endocytosis and initial analysis of these mutants revealed the central role of the actin cytoskeleton and actin associated proteins in the internalization step (Lombardi *et al*., 2001). However, little is known about the exact function of the different proteins involved in endocytosis.

Studies in mammalian cells have identified a complex protein-protein interactions network acting in clathrin-mediated endocytosis (Slepnev and De Camilli, 2000). One of the key player is dynamin, a GTPase shown to be essential for the final pinching off of endocytic vesicles (Hinshaw, 2000). Amphiphysin interacts via its SH3-domain with the PRD-domain of dynamin and is thought to localize dynamin to the clathrin-coated pits (Wigge and McMahon, 1998). Besides these two proteins a large number of other proteins have been implicated in this process and most of them interact with either dynamin, amphiphysin, clathrin or the AP-2 adaptor complex (Slepnev and De Camilli, 2000) but little is known about their exact function. One exception is endophilin, which was recently shown to exhibit lysophosphatidic acid acyl transferase activity and thus might affect the membrane curvature directly (Schmidt *et al*., 1999). Furthermore, phosphorylation was shown to be an important regulatory mechanism for clathrin-mediated endocytosis. The association of an important subset of proteins (e.g. dynamin and amphiphysin) with other components of the endocytic machinery is controlled by coordinated phosphorylation-dephosphorylation cycles (Slepnev *et al*., 1998).

Interestingly, several yeast proteins involved in the internalization step of endocytosis show homology to mammalian proteins implicated in clathrin-mediated endocytosis. For example, Rvs161p and Rvs167p are homologous to amphiphysin and yeast contains also clathrin and AP-2 adaptors. However, clathrin is only partially required for endocytosis in yeast (Payne *et al*., 1988; Tan *et al*., 1993) and AP-2 adaptors are not required at all (Huang *et al*., 1999). Instead, actin is absolutely required in yeast (Kübler and Riezman, 1993) while its involvement in mammalian cells is less clear (Geli and Riezman, 1998). In addition, none of the three yeast proteins with significant homology to dynamin seems to be involved in the internalization step of endocytosis (Geli and Riezman, 1998).

To understand the role of some of the proteins involved in the internalization step of endocytosis in yeast, we looked for protein-protein interactions among them. We found that the yeast amphiphysin homolog Rvs167p interacts with both Sla2p/End4p and Myo5p. Sla2p is an actin-binding protein whose actin-binding domain resides in its C-terminal talin-homology domain (McCann and Craig, 1997). Sla2p was shown to be required for the internalization step of endocytosis and for proper actin cytoskeleton function (Holtzman *et al*., 1993; Raths *et al*., 1993; Wesp *et al*., 1997). Its mammalian homolog Hip1R (Huntingtin interacting protein 1 related) was shown to localize to clathrin coated pits implicating this protein in endocytosis (Engqvist-Goldstein *et al*., 1999). Myo5p is a type I myosin shown to be required for the internalization step of endocytosis and polarization of the actin cytoskeleton (Geli and Riezman, 1996; Goodson *et al*., 1996). Recently, Myo5p together with its homolog Myo3p have been implicated as key players in regulating actin polymerization (Evangelista *et al*., 2000; Geli *et al*., 2000; Lechler *et al*., 2000).

Here, we demonstrate that Rvs167p, Myo5p and Sla2p cofractionate in gel filtration experiments. Furthermore, we could coprecipitate the three proteins from these gel filtration fractions suggesting that they form a protein complex with an approximate size of 600 kDa. We further show that the interaction of the complex with actin is probably mediated via Sla2p and Rvs167p.

Rvs167p has been shown to interact with an increasing number of proteins (Lombardi *et al*., 2001) and therefore these different interactions must be tightly regulated. One obvious regulatory mechanism would be protein phosphorylation. In an accompanying article, Friant and coworkers have identified a pair of homologous protein kinases in yeast, Pkh1p and Pkh2p, that are required for the internalization step of endocytosis. These kinases are the yeast homologs of the mammalian kinase PDK1 (3-phosphoinositide-dependent kinase 1) and were shown to share an essential function (Casamayor *et al*., 1999; Inagaki *et al*., 1999). PDK1 was shown to phosphorylate and thus activate a large set of protein kinases and thereby elicits important physiological responses (Belham *et al*., 1999; Vanhaesebroeck and Alessi, 2000). In a search of the yeast protein database for proteins containing the PDK/Pkh-consensus phosphorylation site we retrieved Rvs167p. Rvs167p has been shown to be phosphorylated by the Pho85 cyclin dependent protein kinase involved in cell cycle regulation (Lee *et al*., 1998). The same study demonstrated that Rvs167p is still phosphorylated *in vivo* in the absence of Pho85 suggesting that other kinases also phosphorylate the protein (Lee *et al*., 1998). The putative PDK/Pkh-phosphorylation site of Rvs167p is localised at the extreme N-terminus of the protein. The N-terminal BAR-domain of Rvs167p was shown to be sufficient to rescue most of the phenotypes of a *rvs167Δ* strain (Colwill *et al*., 1999). We demonstrate that Rvs167p is an *in vitro* substrate for Pkh2p but not for Pkc1p or Ypk1p, two kinases implicated in the Pkh-signaling cascade (Casamayor *et al*., 1999; Inagaki *et al*., 1999). Furthermore, we show that mutation of the phosphorylated Thr7 to Ala affects the internalization step of endocytosis *in vivo*. The same point mutation does not affect the actin cytoskeleton suggesting that the phosphorylation of Rvs167p by Pkh2p is important for the endocytic function of Rvs167p but perhaps not for its actin cytoskeleton function.

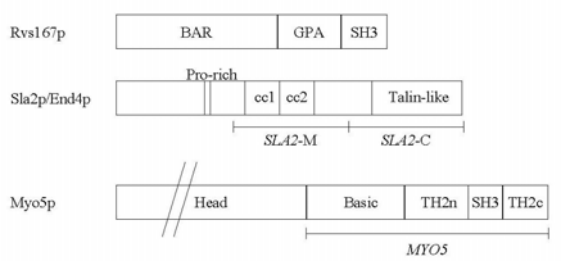

Figure 1. Schematic overview of the domains of Rvs167p, Sla2p/End4p and Myo5p
BAR: BIN/Amphiphysin/RVS domain; GPA: glycine-proline-alanine-rich region; SH3: Src homology 3 domain; Pro-rich: proline-rich region; cc: coiled coil region; Talin-like: Talin homology domain (I/LWEQ module); Head: motor domain of myosins; basic: region rich in basic amino acids; TH2n: N-terminal part of tail homology 2 domain; TH2c: C-terminal part of tail homology 2 domain. *SLA2*-M, *SLA2*-C and *MYO5* show some of the constructs used for the two-hybrid analysis.

Results

Rvs167p interacts with Sla2p/End4p and Myo5p

In order to dissect the internalization step of endocytosis on a molecular level we looked for protein-protein interactions among proteins corresponding to some of the known proteins defective in endocytic mutants. As shown in Figure 2A and 2B we detected a strong interaction of Rvs167p with both Sla2p/End4p and Myo5p in the two-hybrid system. Since the three proteins are built up of different

domains (see Figure 1) we investigated which of these domains are involved in the interactions. We found that the N-terminal BAR-domain of Rvs167p interacted with both the medial and C-terminal parts of Sla2p (Figure 2A). The interaction of Rvs167p with the tail of Myo5p was mediated by either the BAR- or the SH3-domain of Rvs167p (Figure 2B). The TH2n- as well as the SH3-domain of Myo5p were sufficient to mediate the interaction with Rvs167p (Figure 2C).

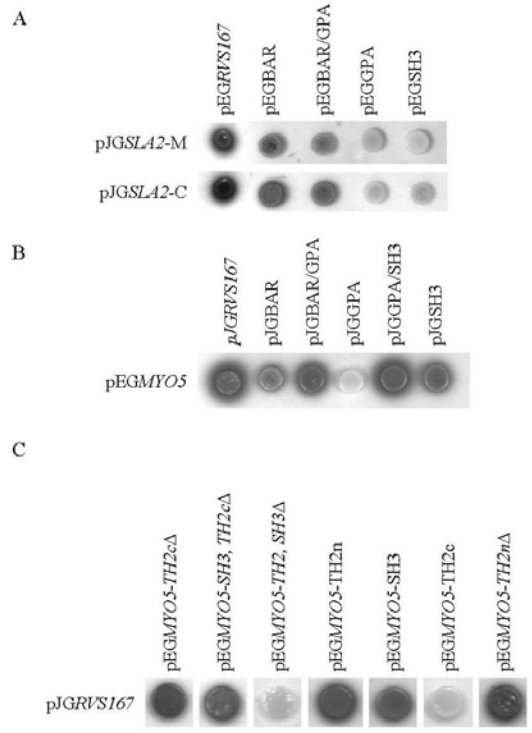

Figure 2. Two-hybrid interactions of Rvs167p with Sla2p and Myo5p
Yeast strain EGY48 containing the reporter gene plasmid pSH18-34 with the lacZ gene under the control of eight LexA operators, a bait, and a prey were streaked out on plates containing X-Gal. Positive interactors turn dark and negative colonies remain white. (A) Rvs167p interacts with Sla2p via its BAR-domain. The central (pJG*SLA2-M*) and C-terminal (pJG*SLA2-C*) parts of Sla2p as a prey were tested against full-length Rvs167p (pEG*RVS167*) and indicated Rvs167p domains as baits. (B) Rvs167p interacts with Myo5p via its BAR- and SH3-domains. The tail of Myo5p (pEG*MYO5*) as a bait was tested against full length Rvs167p (pJG*RVS167*) and indicated Rvs167p domains as preys. (C) Myo5p interacts with Rvs167p via its TH2n- and SH3-domains. Full length Rvs167p (pJG*RVS167*) as a prey was tested against the indicated Myo5p domains as baits.

To confirm biochemically these two-hybrid interactions we constructed a tagged version of Rvs167p on a *CEN*-plasmid (p*RVS167-MYC$_5$-HIS$_6$*). The construct is functional because it rescued the growth defect of a *rvs167Δ* strain on YPUAD + 1M NaCl plates to the same extent as a WT-copy of *RVS167* on a *CEN*-plasmid (p*RVS167*, Figure 3A). We precipitated Rvs167-Myc$_5$-His$_6$p from total cell extracts with Ni-NTA-agarose beads (Qiagen Inc.) and analyzed the pellets by immunoblotting using antibodies for the Myc-epitope, Sla2p and Myo5p. We found that both Myo5p and Sla2p coprecipitated with Rvs167-Myc$_5$-His$_6$p (Figure 3B). These coprecipitations were specific because they occurred only in cells expressing the tagged Rvs167-Myc$_5$-His$_6$p and not in cells expressing WT-Rvs167p. Taken together the two-hybrid and biochemical data demonstrate that Rvs167p interacts with Myo5p and Sla2p.

Rvs167p, Myo5p and Sla2p are part of a 600 kDa protein complex

We next wanted to further characterize the Rvs167p, Myo5p, Sla2p protein complex by performing gel filtration experiments. We loaded total cell extracts on a Superose 6 column, collected 1 ml fractions, TCA precipitated the proteins and analyzed them by SDS-PAGE and immunoblotting. When we used the same strain as for the coprecipitation experiments (RH5237 + p*RVS167-MYC$_5$-HIS$_6$*) we had a very weak signal with the antibodies against Myo5p due to the low amount of protein loaded on the column. Therefore, we decided to use a strain with a Myc-tagged Myo5p (RH5264). As shown in Figure 3C, mycMyo5p was found in fractions 9 to 11, 14 and 15, with the highest amounts in fractions 14 and 15, which was where Sla2p was also found. Rvs167p was found in fractions 8 to 20 with a peak in fractions 14 and 15. These data show that all three proteins

cofractionate in fractions 14 and 15. We detected exactly the same distribution of Sla2p and Rvs167p when we used lysates from the RH5237 + p*RVS167-MYC$_5$-HIS$_6$* strain (data not shown) and therefore used this strain to determine if the three proteins indeed interact with each other in fractions 14 and 15, where they cofractionate. We pooled the fractions 14 and 15, precipitated Rvs167-Myc$_5$-His$_6$p with Ni-NTA-agarose beads and analyzed the pellet by immunoblotting. A strong signal for Myo5p and Sla2p could be detected coprecipitating with Rvs167-Myc$_5$-His$_6$p (Figure 3D) indicating that Rvs167p, Myo5p and Sla2p form a protein complex with an approximate size of 550-600 kDa.

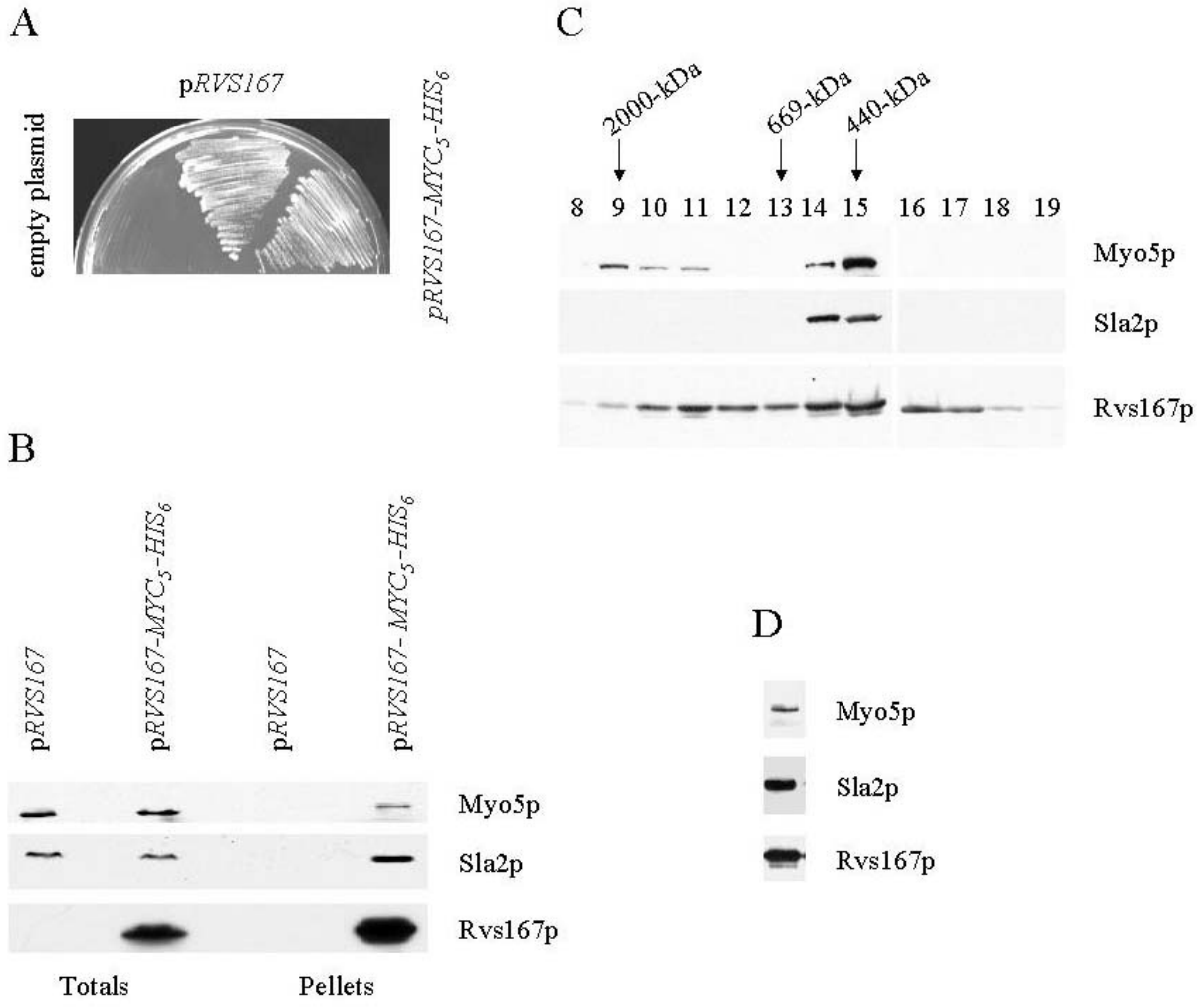

Figure 3. Rvs167p, Sla2p and Myo5p form a 600 kDa protein complex
(A) Rvs167-Myc$_5$-His$_6$p is functional. RH5237 transformed with either Ycplac33 (empty plasmid), p*RVS167* or p*RVS167-MYC$_5$-HIS$_6$* was streaked out on YPUAD + 1 M NaCl plates and grown for three days at 30°C. (B) Coprecipitation of Sla2p and Myo5p with Rvs167p from cell lysates. Total cell lysates from strain RH5237 transformed with either p*RVS167* or p*RVS167-MYC$_5$-HIS$_6$* were incubated with Ni-NTA-agarose beads and the precipitates were analyzed by SDS-PAGE and immunoblotting with antibodies against Myo5p, Sla2p and Myc-epitope (for Rvs167-Myc$_5$-His$_6$p). (C) Gel filtration experiment. Total cell lysates from strain RH5264 were fractionated on a Superose 6 column and the proteins of the different fractions TCA-precipitated and analyzed by SDS-PAGE and immunoblotting with antibodies against Rvs167p, Sla2p and Myc-epitope (for mycMyo5p). (D) Coprecipitation of Myo5p and Sla2p with Rvs167p from gel filtration fractions 14 and 15. Total cell lysates from strain RH5237 + p*RVS167-MYC$_5$-HIS$_6$* were fractionated on a Superose 6 column. Fractions 14 and 15 were pooled, incubated with Ni-NTA-agarose beads and the precipitates were analyzed by SDS-PAGE and immunoblotting with antibodies against Myo5p, Sla2p and Myc-epitope (for Rvs167-Myc$_5$-His$_6$p).

Interaction of the complex with actin is mediated by Rvs167p and Sla2p

Rvs167p, Myo5p and Sla2p were all shown to interact with actin (Amberg *et al*., 1995; Geli *et al*., 2000; McCann and Craig, 1997). However, in the case of Myo5p it has been shown that the interaction with actin is indirect and mediated via Vrp1p (Geli *et al*., 2000).

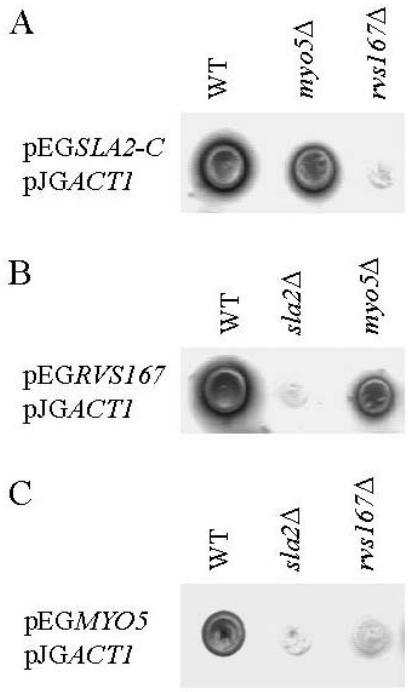

Figure 4. Interaction of the 600 kDa protein complex with actin is mediated by Sla2p and Rvs167p
Yeast strains containing the reporter gene plasmid pSH18-34 with the lacZ gene under the control of eight LexA operators, a bait, and a prey were streaked out on plates containing X-Gal. Positive interactors turn dark and negative colonies remain white. (A) Interaction of Sla2p with actin depends on Rvs167p. The C-terminal part of Sla2p (pEG*SLA2-C*) as bait was tested against full length Act1p (pJG*ACT1*) as a prey in a WT (EGY48), *myo5Δ* (RH5510) and *rvs167Δ* (RH5237) strain. (B) Interaction of Rvs167p with actin depends on Sla2p. Full length Rvs167p (pEG*RVS167*) as bait was tested against full length Act1p (pJG*ACT1*) as a prey in a WT (EGY48), *myo5Δ* (RH5510) and *sla2Δ* (RH5511) strain. (C) Interaction of Myo5p with actin depends on both Rvs167p and Sla2p. Full length tail of Myo5p (pEG*MYO5*) as bait was tested against full length Act1p (pJG*ACT1*) as a prey in a WT (EGY48), *rvs167Δ* (RH5237) and *sla2Δ* (RH5511) strain.

Since we have demonstrated that the three proteins form a protein complex we wanted to determine if the interaction of any of these proteins with actin is affected in the absence of one of the other subunits of the complex. As shown in Figure 4A, the interaction of Sla2p with actin in the two-hybrid system was affected in an *rvs167Δ* but not in a *myo5Δ* strain. Similarly, the interaction of Rvs167p with actin was affected in a *sla2Δ* but not in a *myo5Δ* strain (Figure 4B). Interestingly, the interaction of the tail of Myo5p with actin was affected in both an *rvs167Δ* and a *sla2Δ* strain (Figure 4C). Taken together these data suggest that the interaction of the 600 kDa protein complex with actin requires both Rvs167p and Sla2p.

Rvs167p is phosphorylated at Thr7 by Pkh2p *in vitro*

Rvs167p has been shown to interact with a large number of proteins (Lombardi *et al*., 2001) and we have identified two new ones in this study. It is clear, that these interactions must be regulated and one obvious mechanism could be by protein phosphorylation. Indeed, Rvs167p has been shown to be phosphorylated by the Pho85 cyclin-dependent kinase (Lee *et al*., 1998). In the same study, the authors demonstrated that Rvs167p is still phosphorylated in the absence of Pho85, albeit to a lesser degree, suggesting that other kinases also phosphorylate it. In an accompanying article Friant and co-workers demonstrate that two protein kinases, Pkh1p and Pkh2p, are required for the internalization step of endocytosis. These two kinases are the yeast homologs of mammalian PDK1 (Casamayor *et al*., 1999; Inagaki *et al*., 1999) and a consensus phosphorylation site for these kinases has been determined (see Figure 5; Casamayor *et al*., 1999). By searching the yeast proteins database we found that Rvs167p contains a putative Pkh-phosphorylation site at its extreme N-terminus (Figure 5).

Human proteins (PDK-phosphorylation site)

PKBα	469	F P Q F S
SGK	418	F L G F S
p70 S6Kα	385	F L G F T
PKCζ	691	F R N F S

Yeast proteins (PKH-phosphorylation site)

Ypk1p	658	F G G W T
Ypk2p	655	F G G W T
Pkc1p	1139	F R G F S
Rvs167p	3	F K G F T

Consensus phosphorylation site: FXG[W/F][T/S]

Figure 5. Rvs167p contains a putative PDK/PKH-consensus phosphorylation site
Comparison of the PDK- and PKH-phosphorylation sites in human respectively yeast proteins. Entrez protein database accession numbers: PKBα, XP_015191; SGK, XP_004255; p70 S6Kα, AAA36411; PKCζ, AAA60101; Ypk1p, P12688; Ypk2p, P18961; Pkc1p, AAA34878; Rvs167p, S40887.

Using an *in vitro* phosphorylation assay we could show, that Pkh2p was able to phosphorylate Rvs167p (Figure 6A). The phosphorylation was specific for Pkh2p since Pkc1p and Ypk1p, two protein kinases implicated in the Pkh-signaling cascade (Casamayor *et al*., 1999; Inagaki *et al*., 1999), were not able to phosphorylate Rvs167p *in vitro* (Figure 6A). Since we localized the putative Pkh-phosphorylation site in Rvs167p to amino acids 3 to 7 we wanted to investigate if Thr7 is the residue that is phosphorylated by Pkh2p. We constructed a point mutant, where the Thr7 was changed to an alanine (*RVS167-TA-MYC$_5$-HIS$_6$*) and used this Rvs167-TA-Myc$_5$-His$_6$p as a substrate for Pkh2p in the *in vitro* phosphorylation assay. As shown in Figure 6B, mutant Rvs167-TA-Myc$_5$-His$_6$p was not phosphorylated by Pkh2p. As a further control we used a kinase negative form of Pkh2p (*PKH2-KR*; Inagaki *et al*., 1999) and as shown in Figure 6B, Pkh2-KRp did not phosphorylate Rvs167p. These results show that Pkh2p kinase phosphorylates specifically and directly the yeast amphiphysin homolog Rvs167p.

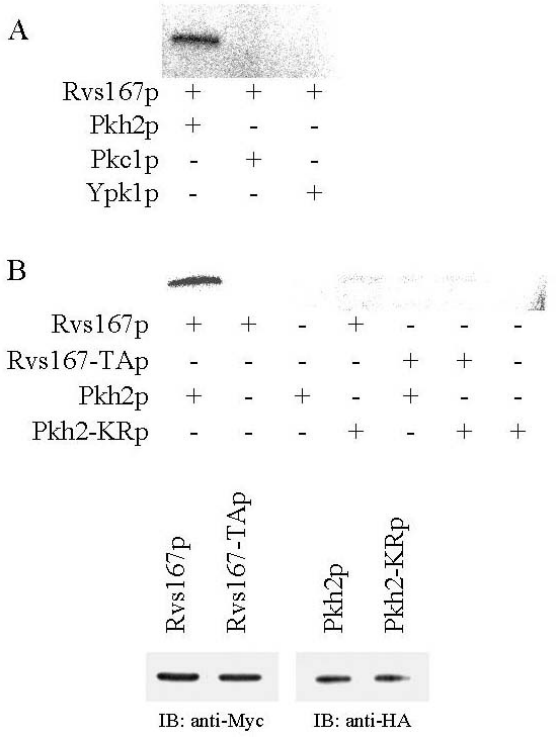

Figure 6. *In vitro* phosphorylation of Rvs167p at Thr 7 by Pkh2p
(A) *In vitro* phosphorylation of Rvs167p by Pkh2p. Yeast strain RH5237 transformed with either p*PKH2*, p*PKC1*, p*YPK1*, p*RVS167-MYC$_5$-HIS$_6$* were grown to log phase. Pkh2-HA$_3$p, Pkc1-HA$_3$p and Ypk1-HA$_3$p were immunoprecipitated from cell extracts. *In vitro* protein kinase assay were conducted with immunoprecipitated Rvs167-Myc$_5$-His$_6$p as a substrate. (B) Thr7 is the phosphorylation site on Rvs167p. Yeast strain RH5237 transformed with either p*PKH2*, p*PKH2-KR*, p*RVS167-MYC$_5$-HIS$_6$* or p*RVS167-TA-MYC$_5$-HIS$_6$* were grown to log phase. Pkh2-HA$_3$p and HA$_2$-Pkh2-KRp were immunoprecipitated from cell extracts. *In vitro* protein kinase assay were conducted with immunoprecipitated Rvs167-Myc$_5$-His$_6$p or Rvs167-TA-Myc$_5$-His$_6$p as a substrate (top). A parallel set of immune complexes was subjected to immunoblotting of Pkh2-HA$_3$p, HA$_2$-Pkh2-KRp, Rvs167-Myc$_5$-His$_6$p or Rvs167-TA-Myc$_5$-His$_6$p (bottom).

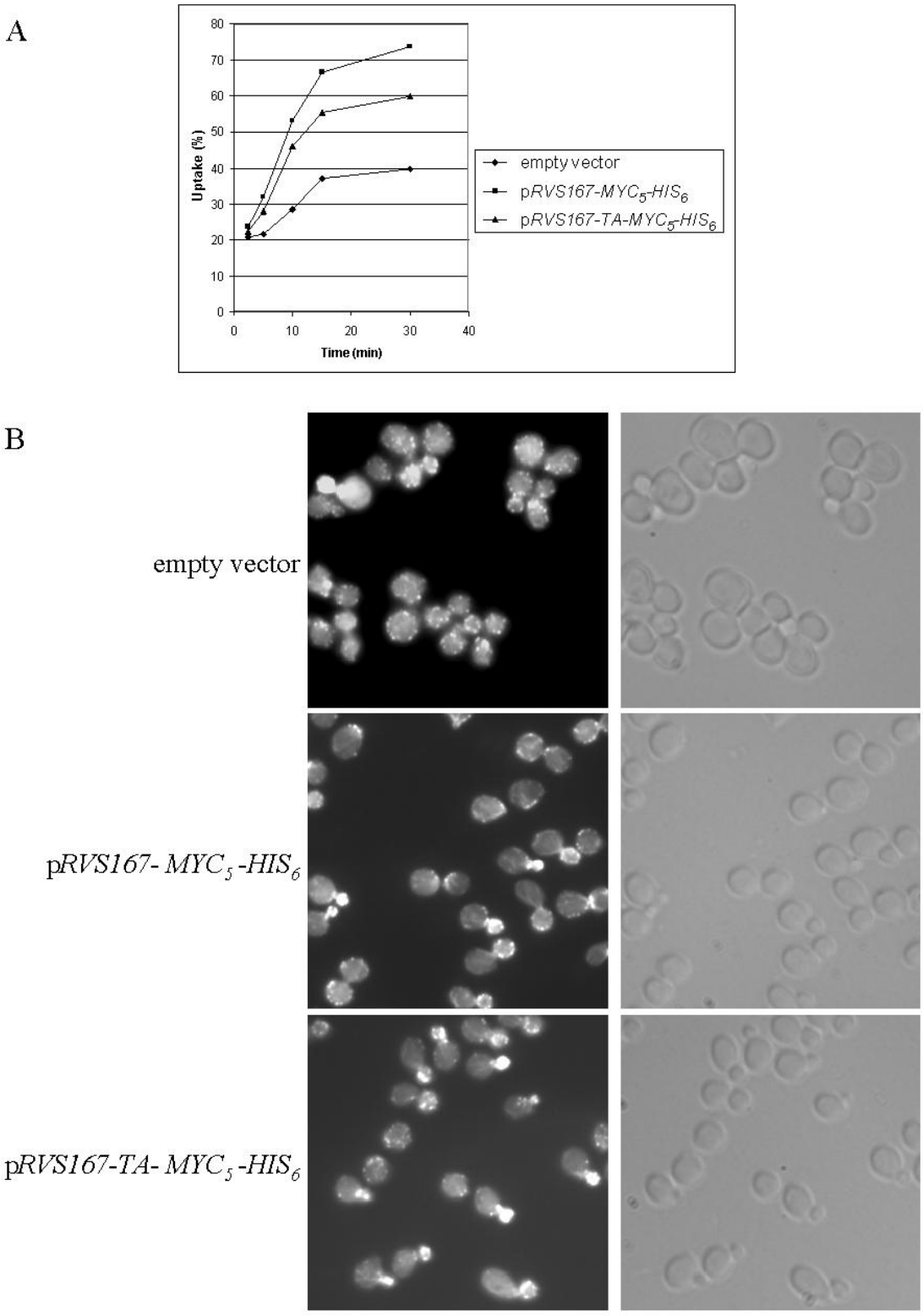

Figure 7. Phosphorylation of Rvs167p at Thr7 is required *in vivo*
(A) Phosphorylation of Rvs167p at Thr 7 is required for the internalization step of endocytosis *in vivo*. Yeast strains RH5262 transformed with either Ycplac33 (empty plasmid), p*RVS167-MYC$_5$-HIS$_6$* or p*RVS167-TA-MYC$_5$-HIS$_6$* were tested for α-factor internalization at 24°C. The values correspond to the average of three independent experiments with standard deviations smaller than 10%. (B) Phosphorylation of Rvs167p at Thr7 is not required for its actin cytoskeleton function. Yeast strains RH5262 transformed with either Ycplac33 (empty plasmid), p*RVS167-MYC$_5$-HIS$_6$* or p*RVS167-TA-MYC5-HIS$_6$* were fixed, and filamentous actin was visualized using TRITC-phalloidin (left panels) and Nomarski optics (right panels).

Phosphorylation of Rvs167p facilitates the internalization step of endocytosis

In a next step, we investigated if the phosphorylation of Rvs167p by Pkh2p is physiologically relevant. To do this, we replaced the wild type *RVS167* with the mutated *RVS167-TA-MYC$_5$-HIS$_6$* allele and assayed its effect on the internalization step of endocytosis. As shown in Figure 7A, we found that the mutated protein was not able to restore the internalization defect of a *rvs167Δ* strain to WT-levels. Instead, we found that the ability of the strain expressing Rvs167-TA-Myc$_5$-His$_6$p to internalize radiolabeled α-factor was significantly less than the strain expressing Rvs167-Myc$_5$-His$_6$p suggesting that phosphorylation at Thr7 is important for endocytosis *in vivo*. Since an *rvs167*Δ strain exhibits a depolarized actin cytoskeleton (Bauer *et al*., 1993) and actin is one of the key requirements for endocytosis in yeast (Kübler and Riezman, 1993) we determined if the mutant Rvs167-TA-Myc$_5$-His$_6$p was also unable to completely restore the actin defect of the deletion strain. As shown in Figure 7B, expression of Rvs167-TA-Myc$_5$-His$_6$p in the *rvs167Δ* strain completely restored the actin defect suggesting that the endocytic defect detected may not be due to a defect in the actin cytoskeleton.

Discussion

In this study we demonstrated that Rvs167p, Sla2p and Myo5p, three proteins required for the internalization step of endocytosis and for actin function, form an approximately 600 kDa protein complex probably involved in regulating the actin cytoskeleton. We showed that the three proteins can be coprecipitated from total cell lysates, that they partially cofractionate in gel filtration experiments and can be coprecipitated from these gel filtration fractions. Using the two-hybrid system we have investigated which domains of the proteins are involved in these interactions. We found that both the central (containing two coiled-coil domains) and the C-terminal (containing the talin-like domain) parts of Sla2p interact with Rvs167p. We have also tested the N-terminal part of Sla2p but we did not detect any interaction with Rvs167p (data not shown). Interestingly, we found that the BAR-domain of Rvs167p interacts with both Myo5p and Sla2p. A previous study demonstrated that the BAR-domain of Rvs167p is sufficient to rescue most of the phenotypes of a *rvs167Δ* strain (Colwill *et al*., 1999) suggesting that the BAR-domain might be the most important domain of Rvs167p. Additionally, we found that the SH3-domain of Rvs167p also interacts with Myo5p. In the case of Myo5p, we showed that either the TH2n- or the SH3-domain were sufficient to interact with Rvs167p. The SH3-domain of Myo5p has been shown to interact with Vrp1p, another protein involved in the internalization step of endocytosis (Anderson *et al*., 1998; Geli *et al*., 2000; Munn *et al*., 1995). Taken together these data suggest a rather complicated interaction pattern among Rvs167p, Myo5p and Sla2p.

Interestingly, a previous study demonstrated that the tail of Myo5p interacts with actin in a physiologically relevant manner and that this interaction is mediated by Vrp1p (Geli *et al*., 2000). Here we find, that in the absence of either Rvs167p or Sla2p the interaction of Myo5p with actin in the two-hybrid system is abolished. One possible explanation could be that the interaction of Myo5p with Vrp1p is stabilized when Myo5p is part of the protein complex with Rvs167p and Sla2p. The interaction of Rvs167p with actin and of Sla2p with actin is affected in absence of Sla2p respectively Rvs167p suggesting that the two interactions stabilize each other. Taken together these data suggest that Myo5p, Rvs167p and Sla2p form an approximately 600 kDa

protein complex stabilized by multiple interactions among the proteins and probably involved in regulating the actin cytoskeleton.

In our gel filtration experiments (Figure 3C) we found, that both Myo5p and Rvs167p were not confined to fractions 14 and 15, while Sla2p was almost exclusively found in these fractions. We could also detect Myo5p in fractions 9 to 11 suggesting that Myo5p is also found in another high molecular weight complex (approximately 2000 kDa). It will be interesting to determine with which proteins Myo5p interacts in this complex since no Sla2p can be detected in these fractions and Rvs167p does not seem to be enriched in these fractions. In contrast to the very distinct distribution of Myo5p and Sla2p in our gel filtration experiments, Rvs167p is more broadly distributed. As already mentioned, Rvs167p has been shown to interact with a large number of proteins (Lombardi *et al*., 2001). Therefore, this broad distribution might reflect the involvement of Rvs167p in several different protein complexes. We have previously shown that Rvs167p needs to interact with its homolog Rvs161p for proper *in vivo* function (Lombardi and Riezman, 2001). Interestingly, we find that the distribution of Rvs161p in the gel filtration experiments closely resembles that of Rvs167p (our unpublished data) supporting the view that the broad distribution of Rvs167p might reflect participation in several complexes rather than just smearing of the protein.

In mammalian cells, amphiphysin has been proposed to function as a scaffold protein that might recruit part of the endocytic machinery including dynamin to sites of endocytosis (Wigge and McMahon, 1998). The yeast amphiphysin homolog Rvs167p might perform a similar function in *Saccharomyces cerevisiae*. As already mentioned, none of the yeast dynamin-homologs is involved in the internalization step of endocytosis (Geli and Riezman, 1998). Instead, the actin cytoskeleton and the motor protein Myo5p are absolutely required (Geli and Riezman, 1996; Kübler and Riezman, 1993) and in this study we have demonstrated that Rvs167p interacts with Myo5p. If we assume that dynamin works as a mechanochemical enzyme in mammalian cells (and therefore provides the driving force to pinch off the endocytic vesicles) and if we take into account that recent studies have demonstrated the involvement of Myo5p in regulating actin polymerization (Evangelista *et al*., 2000; Geli *et al*., 2000; Lechler *et al*., 2000) it is very tempting to put up the following hypothesis. The combined functions of Myo5p as a motor protein and in inducing actin polymerization might be the driving force to pinch off endocytic vesicles in yeast and thus might be the functional equivalent to dynamin in mammalian cells. In agreement with this hypothesis the molecular environments of dynamin and Myo5p are very similar in terms of interacting with amphiphysin/Rvs167p and being part of a complex protein-protein interactions network. Furthermore, recent studies in mammalian cells have shown that endocytic vesicles are associated with actin comet tails (Merrifield *et al*., 1999; Rozelle *et al*., 2000) suggesting that actin polymerization might be involved in pinching off vesicles from the plasma membrane.

In this study we also identified Rvs167p as a target of Pkh2p protein kinase. Pkh2p together with Pkh1p are part of a sphingoid base-mediated signaling pathway that is required for the internalization step of endocytosis (see accompanying article by Friant *et al*.; previous section). Rvs167p is directly and specifically phosphorylated by Pkh2p *in vitro* and could therefore be part of the sphingoid base-activated signaling cascade like Pkc1p, another substrate of Pkh2p. It is worthwhile to point out, that to our knowledge, Rvs167p is the first non-kinase target of the PDK1/2/Pkh1/2p

kinases identified so far. We have demonstrated that Rvs167p is phosphorylated at a specific site (Thr 7) *in vitro* by Pkh2p, but not by two other kinases (Pkc1p and Ypk1p) involved in the Pkh-signaling pathway or in the internalization step of endocytosis like Pkc1p (Friant *et al*., 2000). Mutation of Thr7 to an alanine showed that this mutant Rvs167-TA-Myc_5-His_6p was not phosphorylated anymore by Pkh2p *in vitro*.

The phosphorylation of Rvs167p by Pkh2p is physiologically relevant. Expression of the mutated Rvs167-TA-Myc_5-His_6p in an *rvs167Δ* strain uncovered an endocytic phenotype of the point mutation *in vivo*. Interestingly, we found that the same point mutation was apparently able to completely restore the actin cytoskeleton defect of an *rvs167Δ* strain. However, the immunofluorescence assay for actin function is not a quantitative assay and we could have missed a small defect in Rvs167p function using this assay. Taken together these data demonstrate that the phosphorylation of Rvs167p at Thr7 by Pkh2p is required *in vivo* for the full function of Rvs167p in the internalization step of endocytosis but perhaps not for the actin cytoskeleton function, suggesting that Rvs167p may have a direct role in the internalization step of endocytosis that is independent of its actin function. However, since the endocytic defect seen upon complete loss of Rvs167p is more pronounced than the one seen in the mutant Rvs167-TAp, we must also conclude that the actin cytoskeleton function of Rvs167p is important for endocytosis.

What might be the endocytic function of Rvs167p that is regulated by Pkh2p-dependent phosphorylation? Since we found that the BAR-domain of Rvs167p contains the phosphorylation site and this domain is also sufficient to mediate interaction with both Sla2p and Myo5p, one obvious option would be that the assembly of the protein complex is regulated by this phosphorylation. Using the two-hybrid system we investigated if the Thr7 to Ala mutation affects one these interactions. However, we still detected a strong interaction between Rvs167-TAp and either Myo5p or Sla2p (data not shown) suggesting that the association of Sla2p and Myo5p with Rvs167p may not be regulated by this phosphorylation. However, we cannot rule out the possibility, that association of other proteins to this complex is regulated by phosphorylation of Rvs167p at Thr7. Interestingly, human amphiphysin I also contains a PDK2-phosphorylation site at amino acids 401-405: FNGFT (Entrez protein database accession number XP_004749). The localization of this putative site is different from the one we identified in Rvs167p. However, two recent studies have shown that amphiphysin is a substrate for Cdk5 kinase (Floyd *et al*., 2001; Rosales *et al*., 2000) and Cdk5 is the mammalian homolog of the yeast kinase Pho85 that was shown to phosphorylate Rvs167p (Lee *et al*., 1998). Therefore, both proteins, Rvs167p and amphiphysin, are regulated by homologous kinases, Pho85 and Cdk5, and thus it might well be that amphiphysin I is also regulated by PDK2 as we have shown here for Pkh2p regulation of Rvs167p .

In summary, we demonstrated that Rvs167p is an important player of the endocytic machinery in yeast. We have identified an approximately 600 kDa protein complex containing Myo5p, Rvs167p, Sla2p and probably other unidentified proteins most likely involved in regulating the actin cytoskeleton. Furthermore, we identified Rvs167p as the first non-kinase target for PDK/Pkh-protein kinases and demonstrated that this phosphorylation by Pkh2p is important *in vivo*. We have provided evidence that suggests separation of the function of Rvs167p in the internalization step of endocytosis from its function in regulating the actin cytoskeleton.

Materials and Methods

Yeast strains and media

Yeast strains used in this study are listed in Table I. Strains that did not bear plasmids were grown in complete media YPUAD (2% glucose, 2% peptone, 1% yeast extract, 40 μg/ml uracile, 40 μg/ml adenine and 40 μg/ml tryptophan, 2% agar for solid media). Unless mentioned otherwise, strains bearing plasmids were selected on SD minimal media (Dulic *et al*., 1991).

DNA techniques and plasmid construction

Standard recombinant DNA techniques were used (Sambrook *et al*., 1989). Restriction endonucleases were obtained from MBI Fermentas and Pfu polymerase was obtained from Stratagene. Plasmids used in this study and their relevant features are listed in Table II. Further details are available upon request.

Table I. Yeast strains

Strain	Genotype	Source
EGY48	*Mat **a** his3 trp1 ura3 leu2::lexAop6-LEU2*	(Gyuris *et al*., 1993)
RH5237	*Mat α his3 leu2 trp1 ura3 rvs167::trp1::LEU2 bar1*	this study
RH5262	*Mat **a** his3 leu2 trp1 ura3 rvs167::trp1::LEU2 VRP1-HA$_3$-TRP1 bar1*	this study
RH5264	*Mat **a** ade2 his3 leu2 trp1 ura3 mycMYO5::URA3::myo5::TRP1 bar1*	this study
RH5510	*Mat **a** his3 trp1 ura3 leu2::lexAop6-LEU2 myo5::KanMX6*	this study
RH5511	*Mat α his3 leu2 lys2 trp1 ura3 sla2::his3::LEU2 bar1*	this study

Table II. Plasmids

Plasmid	Yeast Ori	Insert	Source
pEG202	*2μ*	LexA	(Gyuris *et al*., 1993)
pJG4-5	*2μ*	B42	(Gyuris *et al*., 1993)
pSH18-34	*2μ*	8 LexA Op. LacZ	(Gyuris *et al*., 1993)
pEG*RVS167*	*2μ*	*RVS167*	(Lombardi and Riezman, 2001)
pEGBAR	*2μ*	LexA-BAR-domain of *RVS167* (aa 1-281)	this study
pEGBAR/GPA	*2μ*	LexA-BAR- and GPA-domains of *RVS167* (aa 1-423)	this study
pEGGPA	*2μ*	LexA-GPA-domain of *RVS167* (aa 292-423)	this study
pEGSH3	*2μ*	LexA-SH3-domain of *RVS167* (aa 421-482)	this study
pJG*RVS167*	*2μ*	B42-*RVS167*	(Geli *et al*., 2000)
pJGBAR	*2μ*	B42-BAR-domain of *RVS167* (aa 1-281)	(Lombardi and Riezman, 2001)
pJGBAR/GPA	*2μ*	B42-BAR- and GPA-domains of *RVS167* (aa 1-423)	(Lombardi and Riezman, 2001)
pJGGPA	*2μ*	B42-GPA-domain of *RVS167* (aa 292-423)	(Lombardi and Riezman, 2001)
pJGGPA/SH3	*2μ*	B42-GPA- and SH3-domains of *RVS167* (aa 292-482)	(Lombardi and Riezman, 2001)
pJGSH3	*2μ*	B42-SH3-domain of *RVS167* (aa 421-482)	(Lombardi and Riezman, 2001)
pEG*SLA2-C*	*2μ*	LexA-C-terminal part of *SLA2* (aa 665-968)	this study
pJG*SLA2-M*	*2μ*	B42-central part of *SLA2* (aa 334-664)	this study
pJG*SLA2-C*	*2μ*	B42-C-terminal part of *SLA2* (aa 665-968)	this study
pJG*ACT1*	*2μ*	B42-*ACT1*	(Lombardi and Riezman, 2001)
pEG*MYO5*	*2μ*	LexA-*MYO5* tail (aa 757-1219)	(Geli *et al*., 2000)
pEG*MYO5-TH2cΔ*	*2μ*	LexA-*myo5-TH2cΔ* tail (aa 757-1181)	(Geli *et al*., 2000)
pEG*MYO5-SH3,TH2cΔ*	*2μ*	LexA-*myo5-SH3,TH2cΔ* tail (aa 757-1091)	(Geli *et al*., 2000)
pEG*MYO5-TH2,SH3Δ*	*2μ*	LexA-*myo5-TH2,SH3Δ* tail (aa 757-996)	(Geli *et al*., 2000)
pEG*MYO5-TH2nΔ*	*2μ*	LexA-*myo5-TH2nΔ* tail (aa 757-996 + aa 1095-1219)	(Geli *et al*., 2000)
pEG*MYO5*-TH2n	*2μ*	LexA-TH2n-domain of *MYO5* (aa 984-1091)	(Geli *et al*., 2000)
pEG*MYO5*-TH2c	*2μ*	LexA-TH2c-domain of *MYO5* (aa 1142-1219)	(Geli *et al*., 2000)
pEG*MYO5*-SH3	*2μ*	LexA-SH3-domain of *MYO5* (aa 1085-1181)	(Geli *et al*., 2000)
p*RVS167*	*CEN*	pFBKS, *RVS167*	(Bauer *et al*., 1993)
p*RVS167-MYC$_5$-HIS$_6$*	*CEN*	*RVS167-MYC$_5$-HIS$_6$*	this study
p*RVS167-TA- MYC$_5$-HIS$_6$*	*CEN*	*RVS167-MYC$_5$-HIS$_6$* with Thr3 mutated to Ala	this study
p*PKH2*	*2μ*	pTS81, *PKH2-HA$_3$*	Schmelzle and Hall
p*PKH2-KR*	*2μ*	pKT10, *GAL-HA$_2$-PKH2-(K208R)*	(Inagaki *et al*., 1999)
p*PKC1*	*2μ*	pTS94, *PKC1-HA$_3$*	Schmelzle and Hall
p*YPK1*	*2μ*	pTS82, *YPK1-HA$_3$*	Schmelzle and Hall
Ycplac33	*CEN*	no insert	(Gietz and Sugino, 1988)

Two-hybrid analysis

Two-hybrid analysis was performed as described (Geli *et al*., 1998). Briefly, to assay the interaction between a bait and a prey, the strains containing the reporter gene plasmid (pSH18-34), bait and prey were streaked out on plates containing X-Gal. The positive colonies turn dark while the negative ones remain white.

Protein extracts

Strains were grown to exponential phase, harvested and converted to spheroplasts using lyticase (Raths *et al*., 1993). The spheroplasts were lysed by osmotic shock in 1 ml lysis buffer (137 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 10 mM EGTA, and 1 µg/ml of the protease inhibitors aprotinin, leupeptin, pepstatin A, chymostatin and antipain). The protein concentration of the lysates was measured using the Bio-Rad protein assay.

Coprecipitation experiments

Equal amounts of total protein for RH5237 + p*RVS167* and RH5237 + p*RVS167-MYC*$_5$*-HIS*$_6$ were taken, lysis buffer was added to a final volume of 0.9 ml and 100 µl of Ni-NTA-agarose beads (Qiagen Inc.) were added. After two hours of incubation the beads were washed three times with 1 ml lysis buffer + 0.5% NP-40. The precipitates were analyzed by SDS-PAGE and immunoblotting with antibodies against Myo5p, Sla2p and Myc-epitope.

Gel filtration experiments

Protein extracts were loaded onto a Superose 6 gel filtration column (1.0 x 30 cm, Amersham Pharmacia Biotech). The column was developed with running buffer (137 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.5 mM KH_2PO_4) at a flow rate of 0.2 ml/min. 1 ml fractions were collected, the proteins TCA-precipitated and analyzed by SDS-PAGE and immunoblotting with antibodies against Myo5p, Sla2p and Myc-epitope. The molecular mass standards were ferritin (440 kDa), thyroglobulin (669 kDa) and blue dextran (2000 kDa).

For the coprecipitation experiment the fractions 14 and 15 were pooled and the proteins were precipitated as described above.

***In vitro* phosphorylation experiments**

Strains were grown to exponential phase, harvested and lysed with glass beads in buffer (50 mM HEPES pH 7.5, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 150 mM KCl, and 1 µg/ml of the protease inhibitors aprotinin, leupeptin, pepstatin A, chymostatin and antipain). The protein concentration of the lysates was measured using the Bio-Rad protein assay. Equal amounts of total protein for the different kinases and substrates were taken and immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% NP-40, and 1 µg/ml of the protease inhibitors aprotinin, leupeptin, pepstatin A, chymostatin and antipain) was added to a final volume of 0.8 ml. Then 8 µl of antibodies against the HA-epitope (for Pkh2-HA$_3$p, HA$_2$-Pkh2-KRp, Pkc1-HA$_3$p and Ypk1-HA$_3$p) respectively antibodies against Myc-epitope (for Rvs167-Myc$_5$-His$_6$p and Rvs167-TA-Myc$_5$-His$_6$p) and 60 µl of a 30% protein G-sepharose solution were added. After an overnight incubation the beads were washed three times with 1 ml immunoprecipitation buffer, two times with 1 ml buffer (40 mM MOPS pH 7.5) and resuspended in 60 µl phosphorylation buffer (40 mM MOPS pH 7.5, 1 mM DTT, 1 mM $MgCl_2$). For the phosphorylation experiments 10 µl of sepharose beads-substrate immuno-complex (or 10 µl phosphorylation buffer for the control) were incubated with 10 µl

of sepharose beads-protein kinase immuno-complex (or 10 µl phosphorylation buffer for the control) and 3 µCi ^{32}P-γ-ATP for 30' at room temperature. Sample buffer + 50 mM ATP was added and the samples were analyzed by SDS-PAGE and autoradiography.

α-Factor uptakes

α-Factor uptakes (continuous presence) were done as described previously (Dulic *et al*., 1991). The results represent the average of at least three independent experiments.

Actin staining

Yeast cell pre-cultures were grown at 24°C in SD selective media to maintain the plasmids. Cells taken from the pre-culture were then grown at 24°C in YPUAD to early log phase, fixed in formaldehyde and stained with TRITC-phalloidin (Sigma) to visualize F-actin essentially as described previously (Benedetti *et al*., 1994).

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An Intact SH3 Domain Is Required for Myosin I-Induced Actin Polymerization

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Abstract

The yeast type I myosins (*MYO3* and *MYO5*) are involved in endocytosis and in the polarization of the actin cytoskeleton. The tail of these proteins contains a TH2 (Tail Homology 2) domain that constitutes a putative actin-binding site. Because of the important mechanistic implications of a second ATP-independent actin-binding site, we analyzed its functional relevance *in vivo*. Even though the myosin tail interacts with actin and this interaction seems functionally important, deletion of a major portion of the TH2 domain did not abolish interaction. In contrast, we found that the SH3 domain of Myo5p significantly contributes to this interaction implicating other proteins. We found that Vrp1p, the yeast homolog of WIP (WASP Interacting Protein), seems necessary to sustain the Myo5p tail/ F-actin interaction. Consistent with recent results implicating the yeast type I myosins in regulating actin polymerization in vivo, we demonstrate that the C-terminal domain of Myo5p is able to induce cytosol-dependent actin polymerization *in vitro* and that this activity requires both an intact Myo5p SH3 domain and possibly Vrp1p.

My contribution to this work is shown in Figures 1C and 3A.

Introduction

The type I myosins are actin activated ATPases that bear a short positively-charged tail that binds acidic phospholipids and cellular membranes. This feature suggests their role in membrane dynamics (Mooseker and Cheney, 1995; Pollard *et al*., 1991). However, demonstration of their involvement in specific tasks has been difficult. Only recently, genetic approaches have brought some light to this point (Geli and Riezman, 1996; Goodson *et al*., 1996; Jung *et al*., 1996; Novak *et al*., 1995). *MYO3* and *MYO5* encode the type I myosins of the yeast *Saccharomyces cerevisiae*. Deletion of either gene does not result in any obvious phenotype for growth, whereas a double knockout is synthetically lethal or nearly so suggesting functional redundancy (Geli and Riezman, 1996; Goodson *et al*., 1996). The yeast type I myosins are required for the uptake step of endocytosis and the polarization of the actin cytoskeleton (Geli and Riezman, 1996; Goodson *et al*., 1996). However, the mechanistic basis for their function in these processes is unknown.

Besides the putative membrane binding domain (TH1), the tails of Myo3p and Myo5p contain two C-terminal domains that are homologous to other members of this protein family: an Ala and Pro rich domain (TH2) and a Src Homology Domain 3 (SH3 or TH3) (Mooseker and Cheney, 1995; Pollard *et al*., 1991). SH3 domains are present in variety of proteins associated with the organization of the actin cytoskeleton and with signal transduction. This domain mediates protein-protein interactions through binding to proline (Pro) rich stretches (Kuriyan and Cowburn, 1997). The TH2 domain of the protozoal type I myosins binds actin *in vitro* (Brzeska *et al*., 1988; Doberstein and Pollard, 1992; Jung and Hammer, 1994; Rosenfeld and Rener, 1994). However, in contrast to the motor head-actin interaction, ATP does not influence the affinity of the TH2-actin interaction *in vitro*.

A second ATP-independent actin binding site on the type I myosins could have important mechanistic implications. It has been suggested that the TH2 domain could serve to recruit type I myosin molecules onto the actin filaments, securing a high local concentration of myosin heads when the actin/myosin ratio is low and thus it might help to achieve processivity (Albanesi *et al*., 1985). Alternatively, a second actin binding site could allow the type I myosins to crosslink and contract actin filaments (Fujisaki *et al*., 1985; Lynch *et al*., 1986).

Despite the extensive biochemical characterization of the ATP-independent actomyosin interaction of the protozoal type I myosins, its physiological relevance remains obscure. We decided to use site directed mutagenesis on *MYO5* and the Wertman collection of actin alleles (Amberg *et al*., 1995; Wertman *et al*., 1992) to investigate whether the ATP-independent actomyosin interaction is functionally relevant. We find that the Myo5p tail binds F-actin in an ATP-independent manner and this interaction appears to be functionally important. However, in contrast to what was found for the protozoal type I myosins, the SH3 domain of Myo5p significantly contributes to this ATP-independent actomyosin interaction *in vivo* suggesting that the actin binding might not be direct. Consistently, we find that the yeast homolog of the human WIP (WASP Interacting Protein), Vrp1p (Vaduva *et al*., 1999), is required to sustain the SH3-dependent Myo5p tail-actin interaction. Consistent with a recently proposed role of the yeast type I myosins in regulating actin polymerization *in vivo* (Evangelista *et al*., 2000; Lechler *et al*.,

2000) we find that gluthatione sepharose beads coated with a fusion protein consisting of GST and the C-terminal fragment of Myo5p trigger cytosol-dependent actin polymerization *in vitro* and strikingly, such activity seems to require both an intact SH3 domain and Vrp1p.

Results

Functionally relevant interaction between Myo5p tail and actin

The presence of a TH2 domain in Myo5p (Goodson *et al*., 1996) suggests that the Myo5p tail bears an ATP-independent actin-binding site. To investigate this possibility we tested the ability of myc-tagged Myo5p (Geli *et al*., 1998) to pellet with exogenously added F-actin in the presence or absence of ATP. As expected, a significant amount of Myo5p interacted with F-actin in the presence of ATP (Figure 1A). In contrast, a degradation product lacking a C-terminal fragment of about 25 KDa (Figure 1A,*) failed to pellet with F-actin under these conditions. According to the shift in apparent molecular weight, the missing C-terminal fragment should include the TH2 and the SH3 domains. In agreement, a mutant Myo5p lacking these domains (Myo5-(TH2, SH3)Δp) had an apparent molecular weight similar to that of the Myo5p degradation product (*) and failed to pellet with F-actin in the presence of ATP (Figure 1A). A LexA-fusion protein containing only the TH2 and the SH3 domains of Myo5p interacted with F-actin regardless of the presence or absence of ATP in the buffer (Figure 1A, LexA-(TH2,SH3)). These results were confirmed using the two-hybrid assay. The *MYO5* tail (*MYO5*) strongly induced transcription of a β-galactosidase reporter gene when tested against actin (*ACT1*; Figure 1C). Truncation of the Myo5p tail immediately upstream of the TH2 domain abolished the interaction (*MYO5-(TH2,SH3)Δ*) whereas a fragment containing the TH2 and the SH3 domains (TH2,SH3) was sufficient to trigger transcription of the reporter (Figure 1C).

In order to investigate whether the Myo5p tail-actin interaction was functionally relevant, we used a subset of the actin alleles from the Wertman collection (Wertman *et al*., 1992). These actin mutations have been shown to differentially affect interaction with several actin-binding proteins (Amberg *et al*., 1995). Since Act1p and Myo5p are required for the internalization step of endocytosis (Geli and Riezman, 1996; Kübler and Riezman, 1993), the α-factor uptake assay (Dulic *et al*., 1991) can be used to monitor subtle differences in the function of different *ACT1* and *MYO5* alleles *in vivo*. Thus, if the Myo5p tail-actin interaction was functionally important for endocytosis, all actin mutations affecting binding to Myo5p should also exhibit defective uptake kinetics. The opposite assertion is not necessarily true since additional actin binding proteins are necessary for the endocytic uptake. We actually found that all mutations that strongly diminished the interaction with the Myo5p tail severely impaired endocytosis (Figure 2A, *MYO5*). Consistent with this result, the mutant Myo5-(TH2,SH3)Δp, which was unable to pellet with actin in the presence of ATP, was also unable to sustain endocytosis (Figure 2B).

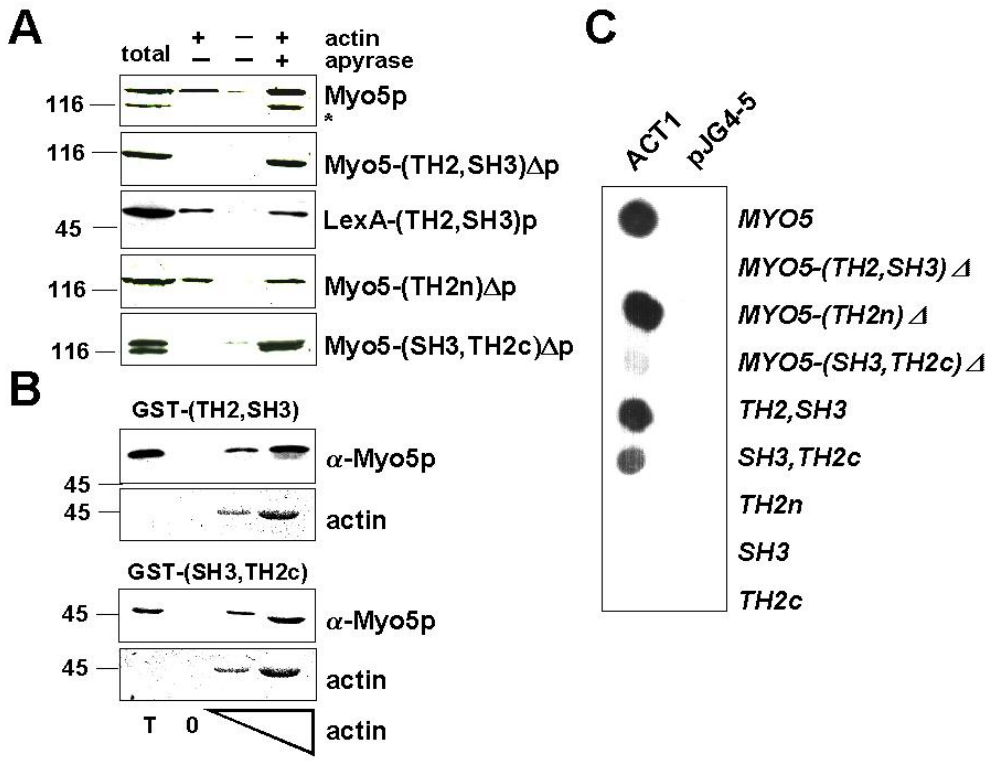

Figure 1. The Myo5p tail interacts with actin
(A) Cytosolic fractions were prepared from strains bearing integrated versions of wt or mutant *myo5* (SCMIG277, SCMIG278, SCMIG279, SCMIG280) or a strain expressing the TH2 and SH3 domains fused to LexA (EGY48 pEG202-*TH2,SH3*) in a buffer containing 1 mM ATP. Purified actin (+ actin) or buffer (- actin) were added to cell extracts. After allowing actin polymerization, one sample containing exogenously added actin was incubated with apyrase to hydrolyze ATP (+ apyrase). F-actin was recovered by centrifugation and the pellet analyzed by immunoblotting. 9E10 was used for detection of Myo5p, and EW/IK for detection of the LexA fusion protein. One quarter of the protein extract utilized per incubation was loaded as total. (B) Recombinant GST fusion proteins (GST-TH2,SH3 or GST-SH3,TH2c) were incubated with cytosolic fractions of a wt strain (RH3975) and increasing concentrations of G-actin. After allowing actin polymerization, F-actin was recovered by centrifugation and the pellets were analyzed for the presence of the recombinant proteins by immunoblotting using the EW/IK antibody. The increasing amounts of actin in the pellet were monitored by Ponceau Red staining. The same amount of GST fusion proteins used per assay were loaded as total. (C) Different *MYO5* tail fragments (pEG202*MYO5*; pEG202*myo5-TH2,SH3Δ*; pEG202*myo5-TH2nΔ*; pEG202*myo5-SH3,TH2cΔ*; pEG202-TH2,SH3; pEG202-SH2,TH2c; pEG202-TH2n; pEG202-SH3; pEG202-TH2c) were tested versus *ACT1* (pJG4-5*ACT1*), or versus the B42 transcriptional activator (pJG4-5) as a control, in the two-hybrid assay on X-Gal-containing plates.

The SH3 of Myo5p contributes to the functionally relevant Myo5p tail-actin interaction

The C-terminal fragment of the Myo5p tail that seems necessary and sufficient to mediate the ATP-independent actomyosin interaction bears both the TH2 and the SH3 domains. The SH3 domain of most protozoal type I myosins is placed at the C-terminus immediately downstream of the TH2 domain (Mooseker and Cheney, 1995). In contrast, the SH3 domain of Myo5p and Myo3p is inserted within the TH2 domain and it splits it into an N-terminal region of about 110 amino acids (TH2n) with more than 40% of Pro and Ala content, and a C-terminal region of about 70 amino acids with only half the percentage of Pro and Ala (TH2c). Deletion of the region encoding the TH2n results in a *MYO5* allele that can

complement the growth defect of a *myo3Δ myo5Δ* mutant and does not display any defect in actin polarization suggesting that this domain is not required for Myo5p function (Anderson *et al*., 1998). In agreement, we found that the analogous mutant could sustain endocytosis with nearly wt kinetics in a *myo3Δ* background (Figure 2B, *myo5-(TH2n)Δ*). Since our data suggested that the Myo5p tail-actin interaction is required to sustain endocytosis we investigated this further. We found that actually, deletion of the N-terminal portion of the TH2 domain neither abolished the ATP-independent actin binding of Myo5p in the pelleting assay (Figure 1A, Myo5-(TH2n)Δp) nor the Myo5p tail-actin interaction in the two-hybrid system (Figure 1C, Myo5-(TH2n)Δp). However, we found that a Myo5p truncated immediately upstream of the SH3 domain no longer pelleted with F-actin in the presence of ATP (Figure 1A, Myo5-(SH3,TH2c)Δp) and its tail only weakly interacted with actin in the two-hybrid system (Figure 1C, *MYO5-(SH3,TH2c)Δ*). Additionally, the C-terminal fragment containing only the SH3 and TH2c domains was able to interact with actin both in the pelleting (Figure 1B) and the two-hybrid assays (Figure 1C). The two-hybrid analysis indicated that both, the SH3 and the TH2c domains, were necessary to sustain the interaction (Figure 1C). Consistent with the functional significance of the (SH3,TH2c)-mediated Myo5p-actin interaction, truncation of Myo5p immediately upstream of the SH3 domain blocked its ability to sustain endocytosis (Figure 2B, *myo5-(SH3,TH2c)Δ*). Additionally, the most C-terminal Myo5p fragment containing the SH3 domain (SH3, TH2c) was sufficient to reproduce the Myo5p tail footprint on actin (Figure 2A).

Vrp1p is required to sustain a physiologically relevant interaction between the Myo5p tail and F-actin

Our data indicates that the SH3 domain of Myo5p might be required to sustain a functionally relevant Myo5p tail-actin interaction. To further test this possibility we analyzed the effect of a point mutation in a conserved residue of the SH3 domain (W1123S). Consistent with the requirement of the SH3 domain in the functionally relevant Myo5p tail-actin interaction, the W1123S mutation prevented binding of the (SH3,TH2c) fragment with actin in the two-hybrid and abolished the interaction between a GST fusion protein containing the TH2 and SH3 domains in the pelleting assays (Figure 3B). This result strongly suggested that the SH3 domain is required for the physiologically relevant Myo5p tail-actin interaction.

The contribution of the SH3 domain to the Myo5p tail-actin interaction implicated that some intermediate proteins might be involved. In an effort to identify the cytosolic components required for the Myo5p tail-actin interaction we tested the need for Vrp1p. Several findings point to the potential role of Vrp1p in this binding event. It was shown that the Myo5p SH3 domain interacts with the proline-rich protein Vrp1p (Anderson *et al*., 1998). On the other hand, Vrp1p, likewise Myo5p, is required for the uptake step of endocytosis and the polarization of the actin cytoskeleton (Munn *et al*., 1995; Vaduva *et al*., 1997) and it interacts with actin in the two-hybrid system (Vaduva *et al*., 1997). Additionally, we observed that the W1123S mutation, that prevented binding of the of the Myo5p C-terminal fragment to actin, also abolished interaction of this domain with Vrp1p in the two-hybrid assay (Figure 3A).

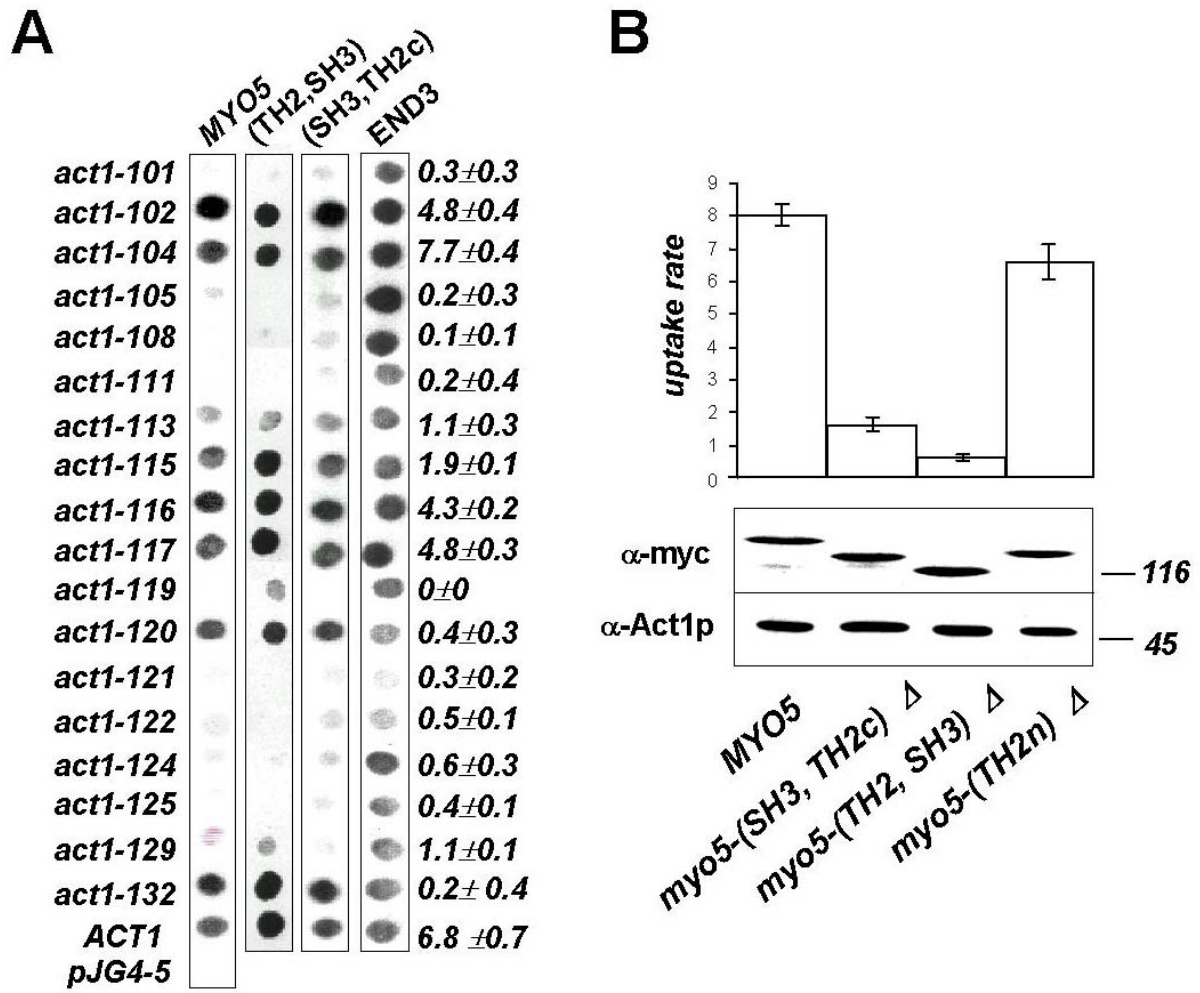

Figure 2. The Myo5p tail-actin interaction is functionally relevant
(A) The indicated *MYO5* tail fragments or *END3* as control (pEG202*MYO5*; pEG202-TH2,SH3; pEG202-SH3,TH2c; pEG*END3*) were tested versus the indicated *ACT1* alleles (pJG4-5*act1-x*) in the two-hybrid X-Gal-containing plate assay. The same actin mutants were tested for their ability to sustain endocytosis *in vivo*. The percentage of cell-bound α-factor internalized/min is indicated. (B) *myo3Δ* strains bearing integrated myc-tagged wt or mutant *MYO5* (SCMIG277, SCMIG278, SCMIG279, SCMIG280) were tested for α-factor internalization. The percentage of cell-bound α-factor internalized/min is indicated. The expression level of the integrated proteins (lower panel) was monitored by immunoblotting. 9E10 was used for detection of wt and mutant Myo5p (lanes 1 - 4). C4 was used for detection of actin as internal reference.

In agreement with a possible requirement of Vrp1p for the Myo5p tail-actin interaction, we observed a strong inhibition of the *MYO5*/*ACT1*-induced β-galactosidase transcription in the *vrp1Δ* strain, when compared to wt cells (Figure 3A). Three criteria indicated that this effect was specific. First, deletion of another protein required for the uptake step of endocytosis and the organization of the actin cytoskeleton, did not significantly affect the Myo5p tail-actin interaction (Figure 3A, *end3Δ*). Second, the interaction of the Myo5p tail with another protein appeared unaffected in the *vrp1Δ* strain (Figure 3A, *MYO5-RVS167*). Third, an N- and a C-terminal truncations of *VRP1*, which were equally unable to complement the growth defect of a *vrp1Δ* strain at 37°C (Vaduva *et al*., 1997), differentially restored the two-hybrid Myo5p tail actin interaction in the *vrp1Δ* cells. The requirement of Vrp1p for the Myo5p tail-actin interaction was confirmed

using the actin pelleting assay. The GST-(SH3,TH2) fusion protein failed to pellet with F-actin when cytosol from a *vrp1Δ* strain was used in the assay (Figure 3C). Also consistent with a proposed role of Vrp1p in mediating the Myo5p tail-actin interaction, HA tagged Vrp1p was also found in the actin pellet together with Myo5p regardless of the presence or absence of ATP in the buffer (Figure 3B). To further test this hypothesis we compare the footprint of Myo5p and Vrp1p on the actin molecule. If Vrp1p was really required to mediate the Myo5p tail-actin interaction, all mutations that diminish Vrp1p-Act1p binding should also impair the interaction of Act1p with the Myo5p tail. As expected, the Myo5p and Vrp1p footprints on actin were strikingly similar (Figure 3C).

An intact SH3 domain and Vrp1p seem to be required for Myo5p-induced localized actin polymerization

Recent results strongly suggest that the yeast type I myosins might be implicated in the regulation of localized actin polymerization *in vivo* (Evangelista *et al*., 2000; Lechler *et al*., 2000). Myo3p and Myo5p physically interact with the Arp2p/Arp3p actin-nucleating complex (Evangelista *et al*., 2000; Lechler *et al*., 2000) and they are required for polarized assembly of cortical actin patches in a semi *in vitro* assay (Lechler *et al*., 2000). On the other hand, Vrp1p has also been implicated in the regulation of actin polymerization *in vivo*. Overexpression of Las17p, the yeast homolog of WASP (Wiscott Aldrich Syndrome Protein), suppresses the actin polarization and endocytic defect of a *ts* allele of *VRP1* but not of a null mutation (Naqvi *et al*., 1998). Las17p interacts with the Arp2p/Arp3p complex and activates its actin nucleation activity *in vitro* (Madania *et al*., 1999; Winter *et al*., 1999). Additionally, a *vrp1Δ* strain is hyper-sensitive to the inhibitor of actin polymerization Latrunculin A and a *vrp1* temperature sensitive (*ts*) mutant can be suppressed by increasing the intracellular concentration of actin by providing an extra copy of *ACT1* (Vaduva *et al*., 1997).

We collected further evidence *in vivo* indicating that Vrp1p and the yeast type I myosins are together involved in positive regulation of actin polymerization and that this function is required for the uptake step of endocytosis in yeast. We previously showed that the *myo5Δ* mutant (but not *myo3Δ* cells) exhibits a partial endocytosis defect at 37°C (Geli and Riezman, 1996) (Figure 4A). Thus, the wt *MYO3* seems to be less efficient than *MYO5* to sustain endocytosis at high temperatures. Interestingly, increasing the intracellular actin concentration by providing an extra copy of *ACT1* could partially rescue the *ts* endocytic defect of the *myo5Δ* strain (Figure 4A). Also, overexpression of Vrp1p efficiently rescued the endocytosis defect of a *myo5Δ* strain. However, over-expression of Vrp1p or Act1p was not able to restore endocytosis in a *myo3Δ myo5-(TH2,SH3)Δ* strain suggesting that suppression required the presence of at least one myosin I-SH3 domain (data not shown). Interestingly, overexpression of Las17p failed to restore endocytosis in the *myo5Δ* strain, suggesting that type I myosins and Las17p only share partially overlapping functions (Evangelista *et al*., 2000; Lechler *et al*., 2000). Additionally, deletion of *MYO5*, but interestingly not deletion of *MYO3*, exhibits synthetic lethality with mutations in genes specifically involved in the regulation of actin polymerization (*ARP3*, *VRP1*) but not with other genes required for polarization of the actin cytoskeleton and endocytosis (i.e. *SLA2*, *RVS161*, *RVS167*, *ABP1*, *SAC6*, *END3*; Figure 4B).

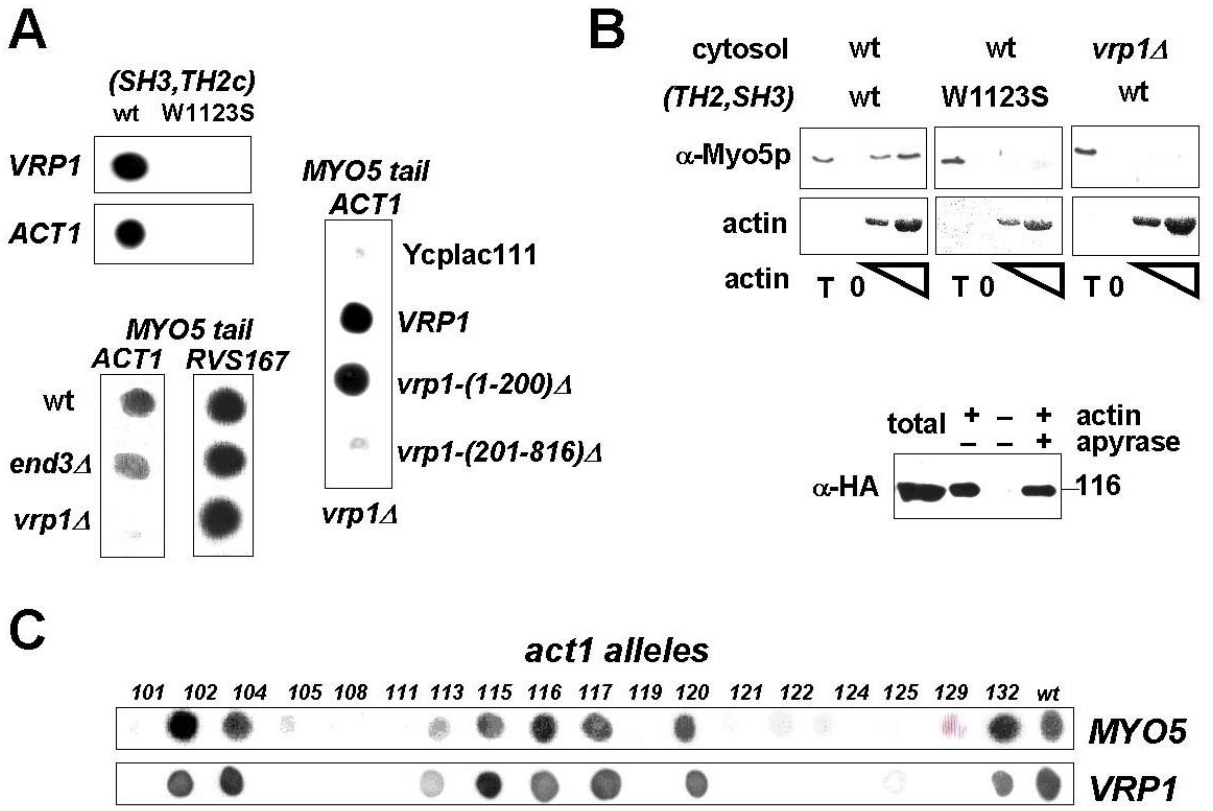

Figure 3. Vrp1p is required for the Myo5p tail-actin interaction
(A) Upper left panel: the most C-terminal *MYO5* fragment containing the SH3 domain (pEG202-SH3,TH2c) wt or the same fragment bearing a point mutation in the SH3 domain (W1223S) was tested versus *ACT1* (pJG4-5*ACT1*) or *VRP1* (pJG4-5*VRP1*) using the two-hybrid assay on X-Gal-containing plates. Lower left panel: the *MYO5* tail (pEG202*MYO5*) was tested versus *ACT1* (pJG4-5*ACT1*) or *RVS167* (pJG4-5*RVS167*) in wt (RH2884) or isogenic *end3Δ* (SCMIG45) or *vrp1Δ* (SCMIG48) strains bearing the β-galactosidase reporter gene (pSH18-34). Enzyme activity was monitored on X-Gal-containing plates. Right panel: the *MYO5* tail (pEG202*MYO5*) was tested versus *ACT1* (pJG4-5*ACT1*) in a *vrp1Δ* (SCMIG48) strain bearing the β-galactosidase reporter gene (pSH18-34), and either an empty plasmid (Ycplac111) or the same plasmid bearing wt or the indicated mutant *vrp1* genes. Enzyme activity was monitored on X-Gal-containing plates. (B) Upper panel: recombinant purified GST fusion proteins containing the wt TH2 and SH3 domains (GST-TH2,SH3) wt, or the same construct bearing the W1223S mutatin in the SH3 domain were incubated with cytosolic fractions from wt (RH3975) or *vrp1Δ* (SCMIG48) strains and increasing concentrations of G-actin. After allowing polymerization, F-actin was recovered by centrifugation and the pellets analyzed for the presence of the recombinant fusion proteins by immunoblotting using the EW/IK antibody. The increasing amounts of actin in the pellet were monitored by Ponceau Red staining. The same amounts of GST fusion proteins used per assay were loaded as total (T). Lower panel: a cytosolic fraction from a strain bearing a plasmid with an HA-tagged *VRP1* (SCMIG277 p181*VRP1HA*) was prepared in a buffer containing 1 mM ATP. Either purified G-actin (+ actin) or G-buffer (- actin) was added to the cell extracts. After allowing actin polymerization, one of the samples containing actin was incubated with apyrase to hydrolyze ATP (+ apyrase). F-actin was recovered by centrifugation and the pellet analyzed by immunoblotting using the anti-HA antibody. One quarter of the protein extract utilized per incubation was loaded as total (T). (C) The *MYO5* tail (pEG202*MYO5*) or *VRP1* (pEG202*VRP1*) was tested versus the indicated *ACT1* alleles (pJG4-5*act1-x*) in the EGY48 strain using the two-hybrid assay on X-Gal-containing plates.

It has recently been pointed out that the C-terminal region of Myo5p shares homology with Las17p including an acidic peptide required for the interaction and activation of the Arp2p/Arp3p complex (Winter *et al*., 1999). Given this information and all previous data regarding the role of Vrp1p and the yeast type I myosins in the regulation of actin polymerization, we hypothesize that Myo3p and Myo5p might be able to induce localized actin polymerization and this activity might then require the interaction with Vrp1p. In order to directly test this hypothesis we established a visual actin polymerization assay *in vitro* based on Ma et al. 1998a and 1998b (Ma *et al*., 1998a; Ma *et al*., 1998b). In this assay gluthatione sepharose beads coated with GST fusion proteins are incubated with cell extracts in the presence of trace amounts of rhodamine-labeled actin. The *de novo* polymerization of actin triggered by a given GST-fusion protein can be monitored under the microscope by visualizing the accumulation of fluorescent signal on the sepharose beads. We chose this assay because, in contrast to the pyrene actin polymerization assay (Cooper *et al*., 1983), it allows to monitor not only actin polymerization but also its localization. Strikingly, we found that beads coated with the GST-(SH3,TH2) fusion protein, but not those coated with naked GST, accumulate a bright fluorescent signal when incubated in cell extracts from a wt strain in the presence of rhodamine labeled actin (Figure 4C). The signal was abolished when the assay was performed in the presence of the Latrunculin A or DNAse I (Figure 4C, table). This result clearly points to a direct role of the yeast type I myosins in the regulation of actin polymerization. Myosin-induced actin polymerization was clearly dependent on the presence of other cellular components since no signal was detected on the GST-(SH3,TH2) coated beads when buffer was used in the assay instead of a cellular extract. Consistent with a proposed function of the yeast type I myosins in the activation of the Arp2/3 complex actin nucleation activity, myosin induced actin polymerization seemed dependent on the presence of the complex since extracts from an *arp2-2* mutant were unable to sustain actin polymerization onto the beads. To investigate a possible requirement of the Vrp1p-Myo5p interaction in this process, we assayed the GST fusion protein bearing the point mutation that abolished interaction of the Myo5p SH3 domain with Vrp1p (W1123S). Interestingly, this construct was unable to trigger accumulation of fluorescently labeled actin onto the coated beads indicating that an intact SH3 domain is required for Myo5p-induced localized actin polymerization. This result also hinted the involvement of Vrp1p in the process. Consistently, we found that cellular extracts from a *vrp1Δ* strain were unable to sustain accumulation of rhodamine-labeled actin onto GST-(TH2,SH3) coated beads (Figure 4C).

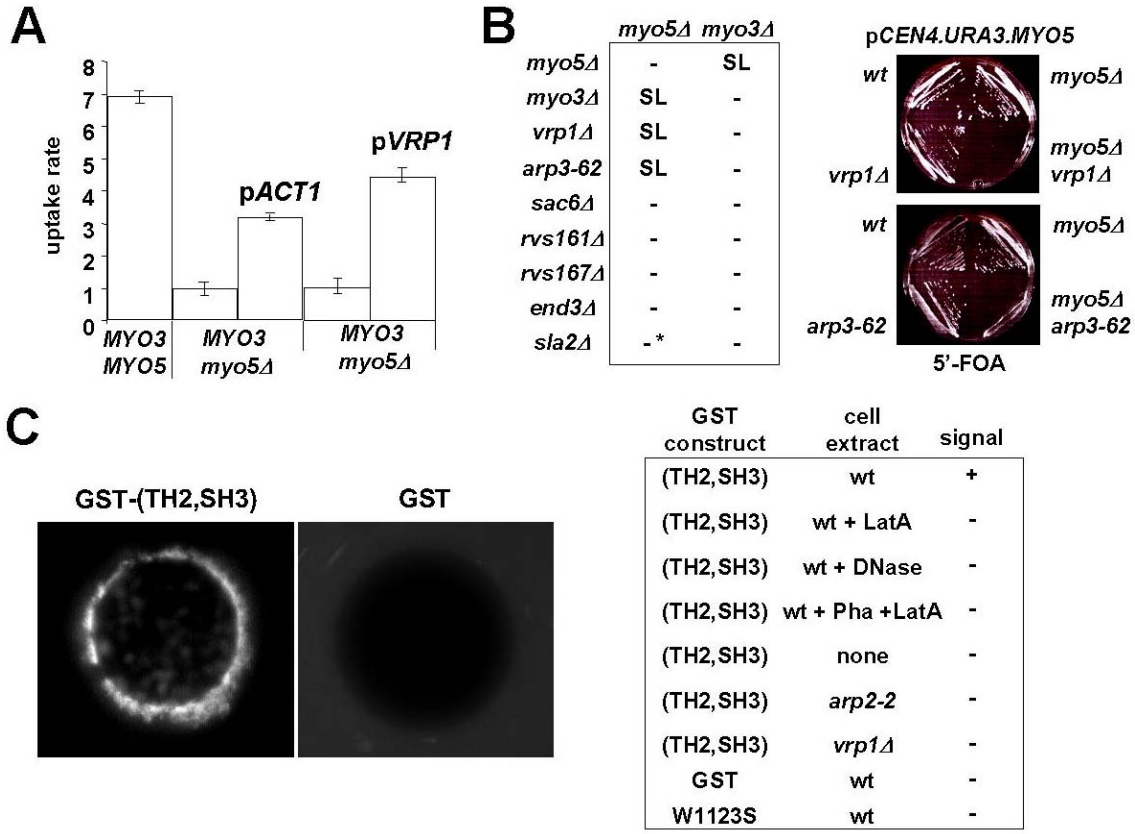

Figure 4. Myo5p might regulate localized actin polymerization
(A) A *myo5Δ* strain (RH3976) bearing plasmids carrying *ACT1* (p413*ACT1*) and *VRP1* (p181*VRP1*) or the corresponding control plasmids (pRS413 and Ycplac181, respectively) was tested for internalization of α-factor. The percentage of cell-bound α-factor internalized/min is shown. A wt (RH3975) was assayed as control. (B) *arp3-62* (SCMIG286) and *vrp1Δ* (RH2892) strains were crossed to *myo5Δ* strains (SCMIG276 and RH3976, respectively) bearing a centromeric plasmid with the *MYO5* and *URA3* genes (p33*MYO5*), to construct the corresponding single or double mutations carrying p33*MYO5*, which were streaked on 5'-FOA plates. A representative of each genotype is shown. Lack of synthetic lethality (SL, see table -) between *myo3Δ* or *myo5Δ* mutations and the indicated mutants (*vrp1Δ*, *arp3-62*, *sac6Δ*, *rvs161Δ*, *rvs167Δ*, *end3Δ* and *sla2Δ*) was assessed by standard tetrad analysis. * Even though the *myo5Δ sla2Δ* spores failed to germinate, the double mutant covered with the p33*MYO5* plasmid was able to grow on 5'-FOA plates. (C) The C-terminal fragment of the Myo5p tail is able to induce actin polymerization *in vitro*. Glutathione-Sepharose beads coated with either GST or GST fused to the *MYO5* TH2 and SH3 domains (GST-TH2,SH3), or the same fusion portein bearing the W1123S mutation, were incubated with extracts from either a wt (RH3975), *vrp1Δ* (SCMIG48) or *arp2-2* (SCMIG4165) strain, or buffer (none), in the presence of rhodamine-actin. Samples were incubated at 30°C and the fluorescent signal visualized using a fluorescence microscope. Latrunculin A (LatA) or Dnase I was added to the samples (wt + LatA and wt + Dnase I, respectively) during the incubation. For the wt + Pha + LatA sample, actin filaments polymerized in the absence of beads were stabilized with phalloidin (Pha) prior to the addition of GST-TH2,SH3-coated beads and latrunculin A. Representative (+) and (-) signals are recorded in the left panel (GST-TH2,SH3 and GST, respectively). The appearance of the beads in a given sample was homogeneous and the assays were repeated at least three times independently.

Discussion

An intact Myo5p SH3 domain is required to sustain a physiologically relevant interaction between the Myo5p tail and F-actin

As previously demonstrated for the protozoal type I myosins that contain a TH2 domain (Brzeska *et al*., 1988; Doberstein and Pollard, 1992; Jung and Hammer, 1994; Rosenfeld and Rener, 1994), we found that the Myo5p tail interacts with F-actin. Despite the extensive biochemical characterization of the second actin-binding site of the protozoal type I myosins, no experiments have addressed its role *in vivo*. We provide here the first genetic evidence suggesting that the Myo5p tail-actin interaction is physiologically relevant. We found that a number of mutations in either *MYO5* or *ACT1* that greatly diminished this interaction, either in the two-hybrid and/or the actin pelleting assays, did also affect their function.

Previous experiments with the protozoal type I myosins indicate that the second ATP-independent actin binding site resides within the TH2 domain (Brzeska *et al*., 1988; Doberstein and Pollard, 1992; Jung and Hammer, 1994; Rosenfeld and Rener, 1994). However, our data indicate that the Myo5p tail-actin interaction detected in either the actin-pelleting and the two-hybrid assays resides within the SH3 and the most C-terminal portion of the TH2 domain. The C-terminal fragment containing the SH3 and the TH2c was sufficient to reconstitute the Myo5p tail footprint on the actin molecule and it was necessary and sufficient to mediate the Myo5p tail-actin interaction both in the two-hybrid and pelleting assays. In contrast, deletion of the N-terminal portion of the TH2 domain did not affect the interaction with actin in either assay. Consistent with this, deletion of the TH2n region in *MYO5* did not disrupt its function in endocytosis or in the polarization of the actin cytoskeleton (Anderson *et al*., 1998). The apparent discrepancy between previous findings for the protozoal myosins and our findings for the yeast type I myosin could be explained in several ways. It could be that the different results reflect differences in the assays used to monitor the interaction. Otherwise, there could be evolutionary divergences in the structural organization of the myosin tails. It is possible that the region defined as a TH2 domain in Myo5p is not a bona fide TH2 domain. Glycines in this region are rare when compared to the protozoal counterparts and the percentage of alanine and proline in the C-terminal region downstream of the SH3 domain is relatively low.

The requirement of the SH3 domain for a physiologically relevant Myo5p tail-actin interaction implicates intermediate proteins in this event. Consistently, we found that a protein that binds both the Myo5p SH3 domain (Anderson *et al*., 1998) and F-actin (This manuscript and Vaduva *et al*., 1997), Vrp1p, seems required to sustain this interaction both in the two-hybrid and in the actin pelleting assays. The functional importance of this interaction is hinted by the demonstration that point mutations in either actin or the SH3 domain of Myo5p, that strongly dimininshed the interaction with Vrp1p, also disrupted the ATP-independent actomyosin interaction and the endocytic function.

The yeast type I myosins might trigger localized actin polymerization at the sites of endocytosis

It was demonstrated recently that in mast cells transfected with a fusion protein consisting of green fluorescent protein (GFP) and β-actin, endocytic vesicles ignite a burst of actin polymerization at the moment they pinch off from the plasma membrane (Merrifield *et al*., 1999). This

result suggests a direct involvement of Apr2/3-dependent localized actin polymerization in some endocytic pathways in mammalian cells. In yeast, mutations in different subunits of the Arp2/3 complex inhibit the uptake step of endocytosis (Moreau *et al*., 1997; Schaerer-Brodbeck and Riezman, 2000a) suggesting that a similar mechanism might be involved. The actin nucleating activity of purified Arp2/3 complex is moderate. It is believed that different molecules, including the members of the WASP family, locally activate the Arp2/3 complex to fulfill distinct cellular functions (Machesky *et al*., 1999; Madania *et al*., 1999; Rohatgi *et al*., 1999; Welch, 1999; Winter *et al*., 1999; Yarar *et al*., 1999). Our results suggest that the yeast type I myosins may play a key role to regulate Arp2/3-dependent actin polymerization at the sites of endocytosis. Surprisingly, we observe that increasing the cellular concentration of actin by providing an extra copy of *ACT1* partially suppresses the *ts* endocytic defect of *myo5Δ* mutant. Further, we could directly demonstrate that the C-terminal domain of Myo5p is able to trigger localized actin polymerization in vitro and such activity most likely depends on the presence of an intact Arp2/3 complex. Type I myosins bind acidic phospholipids with high affinity via their TH1 domain (Mooseker and Cheney, 1995; Pollard *et al*., 1991). Besides, phosphatidic acid and phosphoinositides have been implicated in budding at the plasma membrane (Jost *et al*., 1998; Schmidt *et al*., 1999). Thus, the type I myosins could be recruited to sites where these lipids are locally produced to trigger localized Arp2/3 complex-dependent actin polymerization.

An intact SH3 domain and Vrp1p might be required to localize myosin-induced actin polymerization

The C-termini of Myo3p and Myo5p share homology with the C-terminal acidic peptide of Las17p that greatly activates the actin nucleating activity of purified Arp2/3 complex *in vitro* (Evangelista *et al*., 2000; Lechler *et al*., 2000; Winter *et al*., 1999). In our assay though, the carboxi terminus of Myo5p (TH2c) containing the acidic peptide does not suffice to trigger accumulation of rhodamine-labeled actin (data not shown). Rather, our data clearly indicates that the SH3 domain of Myo5p is also required in this process. A point mutation in the SH3 domain completely abolishes accumulation of fluorescence onto the GST-(SH3,TH2) coated beads. Interestingly, the same mutations also abolished a physiologically relevant interaction of the Myo5p tail with F-actin. A view consistent with all these data is that the SH3 domain of Myo5p is required to bind the actin filaments that are being nucleated by the Arp2/3 complex. Thus, in essence, the SH3 domain would be required to localize actin polymerization rather than to activate nucleation. Since our results indicate that Vrp1p is required to mediate the Myo5p tail interaction with F-actin this hypothesis would be consistent with the observation that a cell extract from a *vrp1Δ* strain is unable to sustain accumulation of rodhamine-labeled actin onto GST-(TH2, SH3) coated beads. This possibility does not exclude that Vrp1p and the carboxi terminal acidic peptide of Myo5p work synergistically to promote actin polymerization. Localized accumulation of F-actin might for example exponentially provide new sites for

activation of the Arp2/3 complex (Blanchoin *et al*., 2000). Additionally, Vrp1p bears a WH2 (WASP homology 2) domain that shares with members of the WASP family including Las17p (Machesky and Insall, 1998; Miki and Takenawa, 1998). This domain binds monomeric actin *in vitro* and has been proposed to promote actin polymerization by increasing the concentration of G-actin in the proximity of growing barbed ends of the filamentous actin.

Materials and Methods

Yeast, media, strains and genetic techniques

Yeast strains used in this report are listed in Table I with their relevant genotypes. Unless otherwise mentioned strains that did not bear plasmids were grown in complete media YPUATD (2% glucose, 2% peptone, 1% yeast extract, 40 μ/ml uracil (Ura), 40 μg/ml adenine (Ade) and 40 μg/ml tryptophan (Trp), 2 % agar for solid media). Strains bearing plasmids were selected on SD minimal media (Dulic *et al*., 1991). Sporulation, tetrad dissection and scoring of genetic markers were performed as described by (Sherman *et al*., 1974). Recombinant lyticase was purified from *E. coli* as described in (Hicke *et al*., 1997). Transformation of yeast cells was accomplished by the lithium acetate method of (Ito *et al*., 1983).

Strains SCMIG277 to SCMIG280 were generated by integrating the corresponding wt and mutant *MYO5* genes as described in (Geli *et al*., 1998). SCMIG37 and SCMIG40 were obtained by crossing RH1995 and RH2892, respectively, to RH2884. Diploids were selected in SD-His and subsequently sporulated, dissected and segregants scored for the appropriate markers.

SCMIG45, SCMIG48 and SCMIG286 were obtained by plating SCMIG37, SCMIG40 and RH4166 respectively, on SD plates containing 2 g/l of 5'-Fluoroorotic acid (5'-FOA). Single colonies were restreaked on SD+5'-FOA plates and all the markers and the *ts* phenotype rechecked. Single colonies with identical phenotype as those of the original strains but unable to grow on SD-Ura, were chosen. SCMIG308 to SCMIG325 were constructed as follows: the heterozygous diploids carrying the different actin alleles linked to *HIS3* (DBY5533, 5534, 5536, 5537, 5538, 5543, 5545, 5546, 5547, 5548, 5550, 5551, 5552, 5553, 5555, 5556, 5559 and 5562, respectively; Wertman *et al*., 1992) were sporulated, dissected and the segregants scored for the different markers. *Mat α act1-x* haploids were then crossed to RH422-8A (*Mat* ***a*** *ACT1 ade2 his3 leu2 ura3 trp1 vma2Δ::LEU2 pVMA2::URA3 bar1*). The diploids were selected on SD-Ura-Leu, sporulated, dissected and the segregants scored for the appropriated markers. *Mat* ***a*** *act1-x his3 leu2 ura3 bar1* and either *ade2* or *ADE2* and *trp1* or *TRP1* were chosen to perform the assays (SCMIG308 to SCMIG325).

DNA Techniques and Plasmid Constructions

All DNA manipulations were performed according to standard techniques (Sambrook *et al*., 1989) unless otherwise specified. Restriction enzymes, Klenow and T4 DNA ligase were obtained from Boehringer Mannheim, New England Biolabs, United States Biochemical or Stratagene Cloning Systems. Plasmids were purified by the Qiagen plasmid purification kit (Qiagen), and transformation of *E. coli* was performed by electroporation (Dower *et al*., 1988). All PCR for cloning purposes were performed with a DNA polymerase with proof reading

activity (Pfu, Stratagene Cloning Systems) and a TRIO-thermoblock (Biometra GmbH). Oligonucletides were synthesized by Microsynth GmbH or Interactiva. All constructs were sequenced by Sequencing service of the ZMBH (Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany).

Plasmids used in this report and their relevant features are listed in Table II. Further details are available under request.

Immunoblots and antibodies

SDS-PAGE for protein separation was performed as described (Laemmli, 1970) using a Minigel system (Bio Rad Laboratories). High and low range SDS-PAGE molecular weight standards (Bio Rad Laboratories) were used for determination of apparent molecular weight. Protein concentration was determined using a Bio Rad Protein assay (Bio Rad Laboratories). Total yeast protein extractions were performed as and Western blots and immunodetection were performed as described in (Geli *et al*., 1998) using the polyclonal EW and IK antibodies against Myo5p C-terminal peptides (Geli *et al*., 1998) the 9E10 anti-myc monoclonal antibody (Roche Biochemicals) and the C4 anti-actin monoclonal antibody (Roche Biochemicals).

Yeast extracts

Yeast cells were grown in rich media to a density of approx. 4 x 10^7 cell/ml. Cells were harvested and washed twice with XB (100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, 1 mM DTT, 1 mM ATP, 10 mM Hepes pH 7.7 and 50 mM Sucrose). 1/10 pellet volume of XB 50 mM Sucrose was added and the cells glass bead lysed in the presence of proteinase inhibitors (0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin). Unbroken cells and debris were eliminated by centrifugation at 2,500 g at 4°C. For low speed pelleted (LSP) extracts, Sucrose was supplemented to 200 mM, freezed in liquid N_2 and stored at –80°C until use in the actin polymerization assay. Usually 30 to 40 mg of protein per ml of LSP extract was measured using the BioRad protein assay. For the high speed pelleted (HSP) or cytosolic extracts, 100 μl aliquots of the LSP extracts were transferred to 1.5 ml polyallomer tubes (Beckman Instruments) and spun twice for 2 h at 45,000 rpm in a TL100 Beckman table top ultracentrifuge using a TLA-45 rotor. Supernatants were then collected, supplemented to 200 mM Sucrose, freezed in liquid N_2 and stored at –80°C until use in the pelleting assays. About 10 to 20 mg of protein per ml of HSP extract was measured using the Bio Rad protein assay.

Recombinant GST fusion protein purification

For purification of recombinant GST fusion proteins, the corresponding pGST plasmids were transformed into BL21 *E. coli* (Novagen). Cultures grown at 24°C in minimal media (MM) (Sambrook *et al*., 1989) supplemented with 50 mg/l Ampicillin were induced at O.D. 0.7-0.8 with 0.1 mM IPTG (isopropylthio-β-D-galactoside) for 2 h. For protein purification, a GST Amersham-Pharmacia purification kit was used (27-4570-01) according to the manufacturer instructions. For the actin pelleting assays, purified GST-fusion proteins were supplemented with 10 % glycerol, freezed in liquid N_2 and stored at –80°C until use. For the actin polymerization assay, GST fusion proteins were freshly purified to a final concentration of 2 mg of fusion protein per ml of 50% glutahion sepharose.

Table I. **Yeast strains**

Strain	Genotype	Reference
RH3375	*Mat**a**/Matα ade2/ADE2 his3/his3 myo3Δ::HIS3/MYO3 myo5Δ::TRP1/MYO5 leu2/leu2 lys2/LYS2 trp1/trp1 ura3/ura3 bar1/bar1*	(Geli and Riezman, 1996)
SCMIG281	*Mat**a**/Matα ade2/ADE2 his3/his3 myo3Δ::HIS3/MYO3 mycMYO5::URA3::myo5Δ::TRP1/MYO5 leu2/leu2 trp1/trp1 ura3/ura3 bar1/bar1*	this study
SCMIG282	*Mat**a**/Matα ade2/ADE2 his3/his3 myo3Δ::HIS3/MYO3 mycmyo5-TH2nΔ::URA3::myo5Δ::TRP1/MYO5 leu2/leu2 trp1/trp1 ura3/ura3 bar1/bar1*	this study
SCMIG283	*Mat**a**/Matα ade2/ADE2 his3/his3 myo3Δ::HIS3/MYO3 mycmyo5-(SH3, TH2)Δ::URA3::myo5Δ::TRP1/MYO5 leu2/leu2 trp1/trp1 ura3/ura3 bar1/bar1*	this study
SCMIG284	*Mat**a**/Matα ade2/ADE2 his3/his3 myo3Δ::HIS3/MYO3 bar1/bar1 mycmyo5-(TH2, SH3)Δ::URA3::myo5Δ::TRP1/MYO5 leu2/leu2 trp1/trp1 ura3/ura3*	this study
RH3975	*Mat**a** ade2 his3 leu2 trp1 ura3 bar1*	(Geli *et al.*, 1998)
RH2881	*Mat**a** his3 leu2 trp1 ura3 bar1*	Riezman laboratory
RH2884	*Matα ade2 his3 leu2 trp1 ura3 bar1*	(Geli *et al*., 1998)
RH3977	*Matα his3 myo3Δ::HIS3 leu2 trp1 ura3 bar1*	(Geli *et al*., 1998)
RH3377	*Mat**a** ade2 his3 myo3Δ::HIS3 leu2 trp1 ura3 bar1*	(Geli and Riezman, 1996)
RH3976	*Mat**a** ade2 his3 myo5Δ::TRP1 leu2 trp1 ura3 bar1*	(Geli *et al*., 1998)
RH3978	*Mat**a** his3 myo5Δ::TRP1 leu2 lys2 trp1 ura3 bar1*	(Geli *et al*., 1998)
SCMIG276	*Matα ade2 his3 myo5Δ::TRP1 leu2 lys2 trp1 ura3 bar1*	this study
SCMIG277	*Mat**a** ade2 his3 myo3Δ::HIS3 mycMYO5::URA3::myo5Δ::TRP1 leu2 trp1 ura3 bar1*	this study
SCMIG278	*Mat**a** his3 myo3Δ::HIS3 mycmyo5-TH2nΔ::URA3::myo5Δ::TRP1 leu2 trp1 ura3 bar1*	this study
SCMIG279	*Mat**a** his3 myo3Δ::HIS3 mycmyo5-(SH3, TH2c) Δ::URA3::myo5 Δ::TRP1 leu2 trp1 ura3 bar1*	this study
SCMIG280	*Mat**a** ade2 his3 myo3Δ::HIS3 mycmyo5-(TH2, SH3) Δ::URA3::myo5 Δ::TRP1 leu2 trp1 ura3 bar1*	this study
RH1995	*Mat**a** his4 leu2 bar1 end3Δ::URA3*	(Bénédetti *et al.*, 1994)
SCMIG37	*Matα ade2 his3 leu2 trp1 ura3 bar1 end3Δ::URA3*	this study
RH2892	*Mat**a** vrp1Δ::URA3 his4 leu2 lys2 ura3 bar1*	(Munn *et al*., 1995)
SCMIG40	*Matα ade2 vrp1Δ::URA3 his3 leu2 lys2 trp1 ura3 bar1*	this study
RH4166	*Mat**a** ade2 arp3-62::LEU2 his3 lys2 leu2 trp1 ura3 bar1::URA3*	B. Windsor
SCMIG286	*Mat**a** ade2 arp3-62::LEU2 his3 lys2 leu2 trp1 ura3 bar1::ura3*	this study
SCMIG273	*Mat**a** las17Δ::LEU2 his3 ura3 leu2 trp1 bar1*	A. Munn
RH3392	*Matα his3 leu2 lys2 trp1 ura3 bar1 end4Δ::HIS3* p*END4::URA3*	(Wesp *et al*., 1997)
SCMIG47	*Matα his3 leu2 lys2 trp1 ura3 bar1 end4Δ::his3::LEU2*	this study
RH2600	*Mat**a** rvs161Δ his4 ura3 bar1*	(Munn *et al*., 1995)
RH2950	*Mat**a** leu2 his4 ura3 trp1::URA3 rvs167Δ::TRP1 bar1*	(Munn *et al*., 1995)
RH2565	*Mat**a** sac6-Δ::URA3 ura3 bar1 his3 leu2*	(Kübler and Riezman, 1993)
RH4165	*Mat**a** arp2-2::URA3 ts GAL+ ade2 trp1 leu2 his lys2 ura3 bar1*	B. Windsor
EGY48	*Mat**a** his3 trp1 ura3 leu2::lexAop6-LEU2*	(Gyuris *et al*., 1993)
SCMIG45	*Matα ade2 his3 leu2 trp1 ura3 bar1 end3Δ::ura3*	this study
SCMIG48	*Matα ade2 vrp1Δ::ura3 his3 leu2 lys2 trp1 ura3 bar1*	this study
SCMIG308	*Mat**a** act1-101::HIS3 ade2 leu2 trp1 ura3 bar1*	this study
SCMIG309	*Mat**a** act1-102::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG310	*Mat**a** act1-104::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG311	*Mat**a** act1-105::HIS3 leu2 ura3 bar1*	this study
SCMIG312	*Mat**a** act1-108::HIS3 leu2 trp1 ura3 bar1*	this study
SCMIG313	*Mat**a** act1-111::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG314	*Mat**a** act1-113::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG315	*Mat**a** act1-115::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG316	*Mat**a** act1-116::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG317	*Mat**a** act1-117::HIS3 ade2 leu2 trp1 ura3 bar1*	this study
SCMIG318	*Mat**a** act1-119::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG319	*Mat**a** act1-120::HIS3 leu2 ura3 bar1*	this study
SCMIG320	*Mat**a** act1-121::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG321	*Mat**a** act1-122::HIS3 ade2 leu2 trp1 ura3 bar1*	this study
SCMIG322	*Mat**a** act1-124::HIS3 ade2 leu2 trp1 ura3 bar1*	this study
SCMIG323	*Mat**a** act1-125::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG324	*Mat**a** act1-129::HIS3 ade2 leu2 ura3 bar1*	this study
SCMIG325	*Mat**a** act1-132::HIS3 ade2 leu2 ura3 bar1*	this study

Actin pelleting assays

The actin pelleting assays performed with the yeast strains expressing the myc tagged wt and mutant Myo5p and HA tagged *VRP1* were designed based on (Wang *et al*., 1998). Cells were grown either in rich media or SD (for plasmid maintenance), to a density of 2 x 10^7 cell/ml. For each pelleting assay, 3 x 10^9 cells were harvested. Half the volume of pellet of 3 x EB was added (EB = 20 mM PIPES pH6.8, 100 mM sorbitol, 100 mM KoAc, 25 mM KCl, 0.5% Triton-X-100, 5 mM

$MgCl_2$, 1 mM ATP, 0.5 mM EGTA, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin) on ice and the cells glass bead-lysed. Unbroken cells and debris were eliminated by centrifugation at 2500 g at 4°C. The supernatants were transferred to a new tube and the protein concentration adjusted to 15 μg/μl with EB. Three 100 μl aliquots of the protein extract were transferred to 1.5 ml polyallomer tubes (Beckman Instruments) and spun 2 h at 45000 rpm in a TL100 Beckman table top ultracentrifuge using a TLA-45 rotor. The supernatants were collected into a fresh tube and 2 x 70 μl were transferred to polyallomer tubes containing 10 μl of 3 x EB and 20 μl of G buffer (5 mM Tris-HCl pH 7.5, 0.1 mM $CaCl_2$, 0.1 mM ATP) containing 2 mg/ml actin (Sigma A-2522). 70 μl were transferred to a polyallomer tube containing 10 μl of 3 x EB and 20 μl of G buffer for the -actin control. After 2 hour incubation at 4°C, 0.2 u of apyrase (Amersham Pharmacia Biotech) was added to one of the samples containing actin for the -ATP control. 10 min after incubation at room temperature (RT), the samples were centrifuged at 45,000 rpm for 2 h at 4°C in the TL100 Beckman tabletop ultracentrifuge using the TLA-45 rotor. The supernatants were discarded and the pellet washed once with 100 μl of EB either treated (for the -ATP control) or not with apyrase. The pellets were resuspended in 30 μl of SDS-PAGE sample buffer and processed for immunoblot analysis. 14 μl of protein extract after the first ultracentrifugation were loaded as total.

For the experiments performed with the recombinant Myo5p fragments, 20 μl of the HSP fraction (adjusted to 10 mg/ml) of the corresponding strain (see above) were diluted with 60 μl XB to bring sucrose concentration to 50 mM, and centrifuged 2 h at 4°C at 45,000 rpm in the TLA-45 rotor. 70 μl of the supernatant was recovered and mixed with 4 μl of G buffer or 4 μl of G-actin either 2 or 4 μg/μl. After mixing, 3 μl of the corresponding GST fusion proteins adjusted to 0.5 μM were added and the samples incubated at 4°C for 2 h. After centrifuging 2 h at 4°C at 45,000 rpm in the TLA-45 rotor, the supernatant was aspirated and the pellet washed with 100 μl of XB 50 mM Sucrose. After centrifuging further 2 h, the supernatant was eliminated and the pellet resuspended in 20 μl of SDS-PAGE SB. 3 μl of the corresponding GST fusion proteins adjusted to 0.5 μM were brought to 20 μl with SDS-PAGE SB and the same amount loaded on the gel as total for comparison.

Visual actin-polymerization assay

The actin polymerization assay was designed according to (Ma *et al*., 1998a; Ma *et al*., 1998b). Briefly, 7 μl of LSP extracts adjusted to 20 mg of protein/ml with XB 200 mM sucrose or just buffer, were mixed with 1 μl of ARS (10 mg/ml Creatine Kinase, 10 mM ATP, 10 mM $MgCl_2$, 400 mM Creatine Phosphate) and 1 μl of 10 μM rhodamine-labeled actin (Cytoskeleton, Inc.). The polymerization reaction was initiated by adding 1 μl of 50% gluthatione sepharose beads bound to 2-3 μg of the corresponding GST fusion protein. Samples were incubated at room temperature (RT) and visualized using fluorescence Microscope Zeiss Axioskop after 10-20 min incubation. Latrunculin and DNase I were added to final concentration of 10 μM and 0.5 μg/μl respectively, previous to the addition of the gluthation sepharose beads.

α-Factor uptake assay

[^{35}S]α-factor uptake assays were performed as described (Dulic *et al*., 1991). A pulse-chase protocol was used. Cells were grown in YPUATD or minimal media at 24°C to a density of 0.5 - 1 x 10^7 cells/ml, harvested

and resuspended in ice-cold YPUATD media for binding of α-factor at 0°C for 45 min. Cells were harvested at 4°C to eliminate unbound α-factor, and internalization was triggered by resuspension in 37°C prewarmed YPUATD. Samples were taken at the indicated time points into pH 1 and pH 6 buffers. Internalized counts were calculated by dividing pH1 by pH6 resistant counts per each time point. The internalization rates were calculated as the percentage of counts internalized per minute between 5 and 10 min (linear range). All uptake assays were performed at least two times and the mean and standard deviations calculated for each time point.

Table II. **Plasmids**

Plasmid	Yeast Ori	Yeast marker	Insert	Reference
Yeplac181	*2 µm*	*LEU2*	-	(Gietz and Sugino, 1988)
p181*VRP1*	*2 µm*	*LEU2*	*VRP1*	this study
p181*VRP13HA*	*2 µm*	*LEU2*	*VRP13HA*	
pRS413	*CEN*	*HIS3*	-	(Sikorski *et al*., 1989)
p413*ACT1*	*CEN*	*HIS3*	*ACT1*	this study
p33*MYO5*	*CEN*	*URA3*	*MYO5*	(Geli *et al*., 1998)
p111*VRP1*	*CEN*	*LEU2*	*VRP1*	this study
p111*vrp1(9–200)Δ*	*CEN*	*LEU2*	*vrp1(9–200)Δ*	this study
p111*vrp1(201–816)Δ*	*CEN*	*LEU2*	*vrp1(201–816)Δ*	this study
pINmyc*MYO5*	-	*URA3*	myc-tagged *MYO5*	this study
pINmyc*myo5-(TH2n)Δ*	-	*URA3*	myc-tagged *myo5-(TH2)Δ*	this study
pINmyc*myo5-(SH3,TH2c)Δ*	-	*URA3*	myc-tagged *myo5-(SH3,TH2c)Δ*	this study
pINmyc*myo5-(TH2, SH3)Δ*	-	*URA3*	myc-tagged *myo5-(TH2, SH3)Δ*	this study
pJG4-5	*2 µm*	*TRP1*	B42	(Gyuris *et al*., 1993)
pJG4-5*ACT1*	*2 µm*	*TRP1*	B42*ACT1*	this study
pJG4-5*act1-101*	*2 µm*	*TRP1*	B42*act1-101*	this study
pJG4-5*act1-102*	*2 µm*	*TRP1*	B42*act1-102*	this study
pJG4-5*act1-104*	*2 µm*	*TRP1*	B42*act1-104*	this study
pJG4-5*act1-105*	*2 µm*	*TRP1*	B42*act1-105*	this study
pJG4-5*act1-108*	*2 µm*	*TRP1*	B42*act1-108*	this study
pJG4-5*act1-111*	*2 µm*	*TRP1*	B42*act1-111*	this study
pJG4-5*act1-113*	*2 µm*	*TRP1*	B42*act1-113*	this study
pJG4-5*act1-115*	*2 µm*	*TRP1*	B42*act1-115*	this study
pJG4-5*act1-116*	*2 µm*	*TRP1*	B42*act1-116*	this study
pJG4-5*act1-119*	*2 µm*	*TRP1*	B42*act1-119*	this study
pJG4-5*act1-120*	*2 µm*	*TRP1*	B42*act1-120*	this study
pJG4-5*act1-121*	*2 µm*	*TRP1*	B42*act1-121*	this study
pJG4-5*act1-122*	*2 µm*	*TRP1*	B42*act1-122*	this study
pJG4-5*act1-124*	*2 µm*	*TRP1*	B42*act1-124*	this study
pJG4-5*act1-125*	*2 µm*	*TRP1*	B42*act1-125*	this study
pJG4-5*act1-129*	*2 µm*	*TRP1*	B42*act1-129*	this study
pJG4-5*RVS167*	*2 µm*	*TRP1*	B24*RVS167*	this study
pEG202	*2 µm*	*HIS3*	LexA	(Gyuris *et al*., 1993)
pEG202*MYO5*	*2 µm*	*HIS3*	LexA*MYO5* tail (aa 757–1219)	this study
pEG202*myo5-(TH2c)Δ*	*2 µm*	*HIS3*	LexA*myo5-(TH2c)Δ* tail (aa 757–1181)	this study
pEG202*myo5-(SH3,TH2c)Δ*	*2 µm*	*HIS3*	LexA*myo5-(SH3,TH2c)Δ* tail (aa 757–1091)	this study
pEG202*myo5-(TH2, SH3)Δ*	*2 µm*	*HIS3*	LexA*myo5-(TH2, SH3)Δ* tail (aa 757–996)	this study
pEG202*(TH2, SH3)*	*2 µm*	*HIS3*	LexA*(TH2, SH3)* domain (aa 984–1219)	this study
pEG202*(SH3, TH2c)*	*2 µm*	*HIS3*	LexA*(SH3, TH2c)* domain (aa 1085–1219)	this study
pEG202*(TH2n, SH3)*	*2 µm*	*HIS3*	LexA*(TH2n, SH3)* domain (aa 984–1181)	this study
pEG202*TH2c*	*2 µm*	*HIS3*	LexA*TH2c* domain (aa 1142–1219)	this study
pEG202*TH2*	*2 µm*	*HIS3*	LexA*TH2* domain (aa 984–1091)	this study
pEG202*SH3*	*2 µm*	*HIS3*	LexA*SH3* domain (aa 1085–1181)	this study
pEG202*myo5-TH2nΔ*	*2 µm*	*HIS3*	LexA*myo5-TH2nΔ* tail (aa 757–996) + (aa1095–1219)	this study
pEG202*END3*	*2 µm*	*HIS3*	LexA*END3*	this study
pSH18–34	*2 µm*	*URA3*	8 LexA Op. LacZ	(Gyuris *et al*., 1993)
pRFM-1	*2 µm*	*HIS3*	LexAbicoid	(Gyuris *et al*., 1993)
pGEX5X-3	-	-	GST	
pGST-*(TH2,SH3)*	-	-	GST-*(TH2, SH3)* domain (aa 984–1219)	this study
pGST-*(TH2,SH3)*-W1123S	-	-	GST-*(TH2, SH3)* domain (aa 984–1219)–W1123S	this study
pGST-*(SH3,TH2c)*	-	-	GST-*(SH3, TH2c)* domain (aa 1085–1219)	this study
pGST-*(TH2c)*	-	-	GST-*TH2c* domain (aa 1142–1219)	this study

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Conclusions and Perspectives

Some thoughts about "complexity"

The last couple of years have brought an enormous increase in the number of organims whose genomes have been sequenced. Scientific "Pet" organisms like *S. cerevisiae*, *D. melanogaster* and *C. elegans* have been sequenced and last but not least also a working draft of the *H. sapiens* genome is available. Using sophisticated gene-prediction software the approximate number of genes encoded by the different genomes was determined and of course the different species were compared. To the big dissapointment of the general public and especially of pharmaceutical and biotechnology companies, the latest studies predict only about 30'000 to 40'000 genes for humans and not 70'000 to 100'000 as some previous studies had predicted. There is a big disappointment that humans are only about two times as "complex" as worms and only about five times as "complex" as yeast even though no one can really say what the term "complex" really means. Fortunately, the focus seems to turn away from the mere number of genes to what genes are actually for: they mostly encode for proteins! DNA is very important because it encodes almost the whole properties of the cell (it is believed that some membrane properties of organelles are "inherited" to "daughter" organelles and cannot arrise *de novo*). However, the cell is mainly made of proteins and lipids and proteins carry out most of the activities going on in a cell. Different cell types of the same organism express different proteins even though they have exactly the same genome. To further complicate the whole system one gene can encode for more than one protein because alternative splicing and RNA editing can produce from one precursor mRNA a large number of "mature" mRNA's encoding for different proteins leading to an increase in complexity. However, an increased number of proteins per se does not necessarily mean increased "complexity". What really determines the "complexity" of a cell are the interactions among its proteins and lipids that allow a cell to function properly. The regulation of protein-protein interactions networks and the integration and fine-tuning of outputs from the diverse networks are the key features that ensure survival and allow a cell to pursue its tasks. If we want to understand the smallest "unit of life" (i.e. a cell) we need to understand how it works at a molecular level by unravelling its functional networks. Anyways, the existence of life is such a wonder that the classification in different classes of "complexity" is pure semantics. Especially because every single living organism has proven to be the best suited for its place in nature under the current conditions.

Protein-protein and protein-lipid interactions network

In this PhD thesis we have started to unravel part of the interactions network involved in the internalization step of endocytosis on a molecular level. As previously described in the introduction, the different proteins required for this process can be distributed to different protein complexes based on genetic and/or biochemical data. However, recent studies from several groups (see introduction) and work presented here in this thesis have clearly demonstrated that the described protein complexes are much more interconnected than previously thought leading to the notion that the whole system is a complex network of interactions. (see Figure 1). To further increase the complexity, lipids add an additional level of regulation and seem to work via different mechanims to allow correct working of the process. Sterols might provide the proper membrane environment to allow the endocytic machinery to be recruited and/or to assemble at specific sites. Another possible function might be to modulate the biophysical properties of the membrane to allow the proper membrane

curvature to build and/or to allow an endocytic vesicle to pinch off. In contrast, sphingoid bases seem to act as signaling molecules controling a whole cascade of protein kinases starting with Pkh1p and Pkh2p and required for the internalization step (this thesis). Previous data (Friant *et al*., 2000) and work presented in this thesis suggest that one of the roles of this sphingoid base-mediated signaling cascade might be to regulate the actin cytoskeleton. Our initial studies have identified two possible effectors of this signaling pathway (Rvs167p and Pkc1p) and we can assume that there will be more (e.g. Yck2p). Therefore we propose that this signaling cascade will affect endocytosis directly and not only via regulating the actin cytoskeleton.

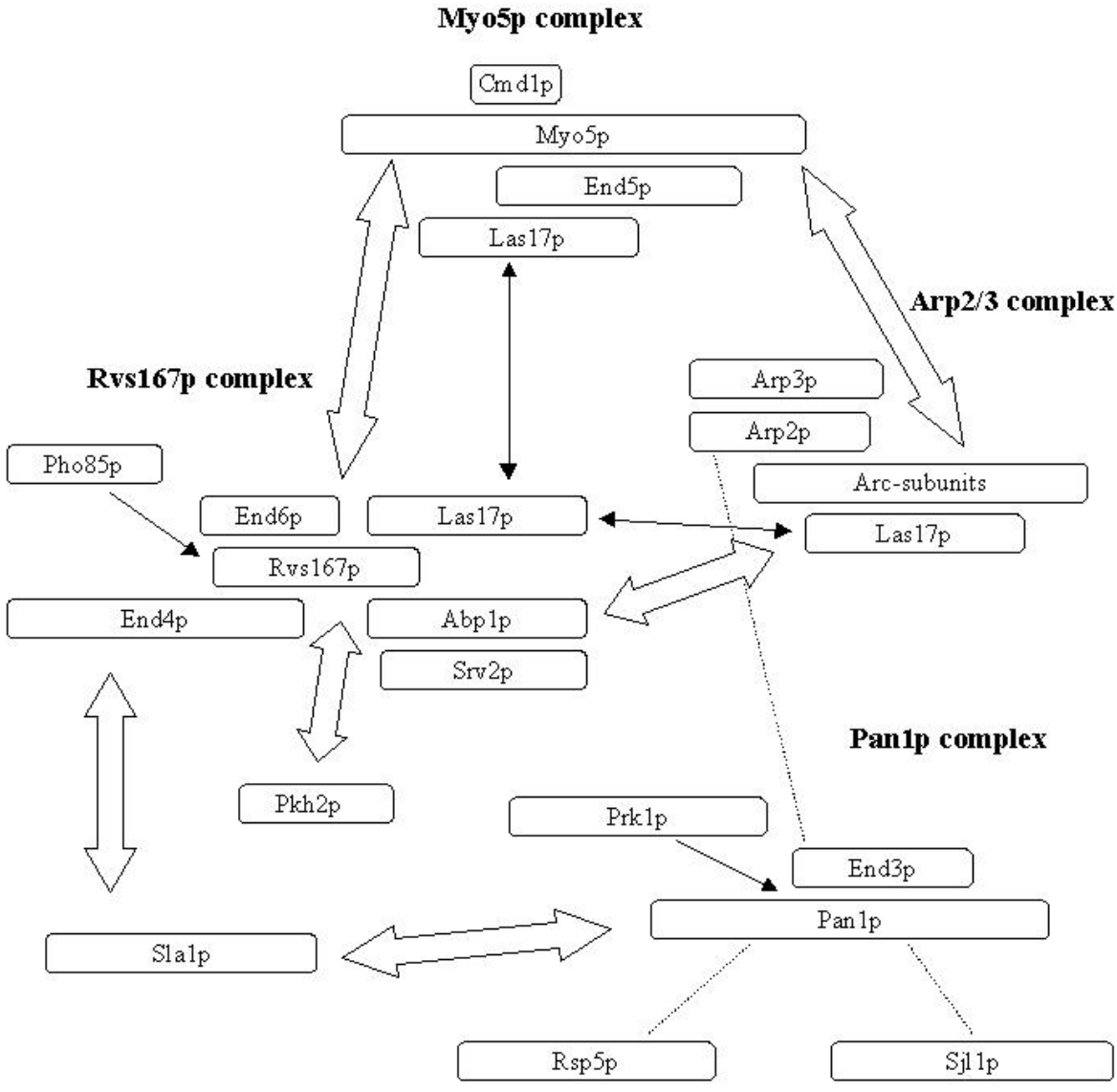

Figure 1. Protein-protein interactions network involved in the internalization step of endocytosis
Schematic drawing of the new connections (big double-headed arrows) between the four protein complexes described in detail in the introduction.
Proteins that have been shown to interact biochemically and/or in the two-hybrid system are drawn near each other. Synthetic lethal interactions are shown by a dashed line, and an arrow marks phosphorylation of a protein by a kinase. Las17p might be part of three complexes and therefore connections are drawn by double-headed arrows.

We have provided preliminary data suggesting that the phosphorylation of Rvs167p by Pkh2p is required for its endocytic function but maybe not for its actin function (see third section of the results). However, we cannot exclude that the immunofluorescence assay used to monitor the actin cytoskeleton is not sensitive enough to monitor subtle changes. Furthermore, we do not know which actin structures are really required for endocytosis. Interestingly, recent studies have identified two new proteins, Myo5p and Abp1p, that activate the actin nucleation functions of the Arp2/3 complex (Evangelista *et al*., 2000; Goode *et al*., 2001; Lechler *et al*., 2000; this thesis). Myo5p has been shown to be involved in the internalization step (Geli and Riezman, 1996) and Abp1p has a redundant endocytic function with the central coiled-coil domains of Sla2p (Wesp *et al*., 1997). Therefore, both proteins could be involved in initiating and/or regulating the actin structures required for internalization. However, in the case of Abp1p the *in vivo* relevance of its role in Arp2/3 complex acitvation has not been thorougly adressed, yet. In the case of Myo5p the evidence supports a physiologically relevant interaction with actin.

Rvs167p is one of the two yeast amphiphysin homologs. Members of this protein family have been implicated in endocytosis. Work presented in this thesis and several other studies have suggested a high degree of similarity in terms of regulation and binding partners between Rvs167p and amphiphysin (Floyd *et al.*, 2001; Lee *et al*., 1998; Lombardi and Riezman, 2000; Navarro *et al*., 1997; Rosales *et al*., 2000; Wigge *et al*., 1997). Amphiphysin I interacts with amphiphysin II as does Rvs167p with Rvs161p. Rvs167p is phosphorylated by Pho85 kinase as is amphiphysin by the mammalian Pho85 homolog Cdk5 suggesting that at least part of the regulation has been conserved through evolution. We have shown in this thesis that Pkh2p phosphorylates Rvs167p and have identified a PDK2 consensus phosphorylation site in amphiphysin I. Given the striking similarities between Rvs167p and amphiphysin I we propose that PDK2 might phosphorylate and thus regulate amphiphysin I. Furthermore, it might be interesting to investigate a possible role of PDK-kinases in clathrin-mediated endocytosis in mammalian cells.

Outlook

It is very clear, that we have just started to unravel the interactions network acting at the internalization step of endocytosis. There are still a large number of proteins missing and even more protein-protein interactions to be discovered. However, what is almost completely missing is knowledge about the actual functions of the proteins involved in this process.

Up to now, the type I myosin Myo5p was the only protein where a direct role in force generation and/or rearrangement of actin filaments could be postulated. However, now several studies (Evangelista *et al*., 2000; Lechler *et al*., 2000; this thesis) have unravelled an additional function of Myo5p in actin polymerization suggesting that Myo5p might not behave as a normal motor protein carrying its cargo from A to B. Myo5p might be able to induce the actin filaments it needs to move and/or generate additional force by polymerizing new actin filaments at the sites of internalization by activating the Arp2/3 complex. As mentioned previously, we don't know which actin structures are involved in the internalization step. However, the actin patches are most likely not the sites of internalization (Mulholland *et al*., 1999) suggesting that a more specialized actin structure not visible by conventional fluorescence microscopy might be required.

For the rest of the proteins we don't have any concrete idea about their role in internalization. Most of them affect the actin cytoskeleton and therefore it would be interesting to determine if their endocytic defect is solely due to the actin defect or if they have a more direct role in the process. One obvious approach would be to generate mutants and determine if the two phenotypes can be genetically separated. Furthermore, one could determine if they work on actin directly by investigating how they affect actin polymerization and/or the stability of F-actin.

However, the most usefull tool to understand the role of the different proteins in the internalization process would be an *in vitro* endocytosis assay. This would allow to dissect at which step a certain protein is involved by interfering with the protein function and determining where the process is blocked. Furthermore, the minimal requirements to build and pinch off an endocytic vesicle might be determined giving a hint which factors are absolutely necessary, which factors increase the efficiency and which factors are involved in regulation.

Another major issue is to understand how internalization is regulated. There are now several kinases identified to be required for the internalization step (Pkh1p, Pkh2p, Pkc1p and Yck2p; Friant *et al*., 2000; this thesis). Two other kinases, Ark1p and Prk1p, have been shown to be involved in regulating the actin cytoskeleton and might be linked to the Pan1p complex (see introduction, section 3.2.4.). However, there is no report about a possible role in endocytosis. Sphingoid bases have been shown to activate a whole signaling cascade including at least three of these kinases (Pkh1p, Pkh2p and Pkc1p; Friant *et al*., 2000; Zanolari *et al*., 2000; this thesis). We have identified Rvs167p as a downstream effector of Pkh2p but we don't know what targets Pkc1p regulates. Furthermore, we don't know if Yck2p is also part of this sphingoid base-mediated signaling cascade or if it defines another signaling pathway involved in regulating internalization.

We are far away from understanding the internalization step of endocytosis at a molecular level but we have started to design a rough sketch on which to build up.

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