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Diplom-Biologin Bianka Lucretia Großhans

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Regulation of Myo5p function by phosphorylation

Gutachter: Prof. Dr. Felix Wieland

Prof. Dr. Bernhard Dobberstein

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1 Summary

The *Saccharomyces cerevisiae* type I myosins Myo3p and Myo5p are involved in endocytosis and in the polarization of the actin cytoskeleton. *In vitro*, p21-activated kinases (PAKs) can phosphorylate a single serine in the myosin-I head domain, referred to as TEDS site, thereby activating the myosin-I ATPase.

This work demonstrates that phosphorylation of the Myo5p TEDS site is required for two myosin-I *in vivo* functions in yeast, endocytosis and actin cytoskeleton polarization. However, the yeast PAKs Ste20p, Cla4p and Skm1p and their activator Cdc42p, while important for actin cytoskeleton polarity, are not required for the uptake step of endocytosis. An *in vitro* screen identified several other kinases as possible TEDS site kinases including the sphingoid base-activated kinase Pkh2p. Preliminary results support a model in which Pkh2p and its paralogue Pkh1p are part of a signalling cascade that leads to TEDS site phosphorylation and thus myosin-I activation for endocytosis.

A second important feature of fungal myosins-I is their ability to induce actin polymerization through the Arp2/3 complex. Within this work, Myo5p serine 1205, located N-terminal to the sequence that interacts with and activates the Arp2/3 complex, was identified as a phosphorylation site for casein kinase II (CKII). This phosphorylation event appears to negatively regulate myosin-I-induced actin polymerization. The fact that mutation of one catalytically active subunit of CKII increases the α-factor internalization rate, points to a inhibitory role of this kinase in endocytosis, which might be a direct consequence of the inhibitory effect of myosin-I tail phosphorylation on Arp2/3 dependent actin polymerization.

2 Abbreviations

1NM-PP1	4-amino-1-tert-butyl-3-(1-naphtylmethyl) pyrazolo [3,4-d] pyrimidine
aa	amino acid
ADP	adenosine 5'-diphosphate
Amp^r	ampicillin resistance gene
as	analogue-sensitive
ATP	adenosine 5'-triphosphate
bp	base pairs
Ci	Curie
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
g	gravity
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IgG	immunoglobulin G
kDa	kilodalton
KPi	potassium phosphate
MOPS	(3-[N-Morpholino]propanesulfonic acid)
OD_{600}	optical density at 600nm
ORF	open reading frame
PCR	polymerase chain reaction
ProtA	protein A of *Staphylococcus aureus*
RT	room temperature
SDC	synthetic dextrose complete medium
TRIS	tris-(hydroxymethyl)-aminomethane
ts	temperature-sensitive
U	unit
WT	wild-type
YPD	yeast peptone dextrose medium

3 Introduction

The microtubules, the actin filaments and the intermediate filaments compose the cellular cytoskeleton. The cytoskeleton establishes cell shape, resists mechanical deformation and serves as tracks along which motor proteins can deliver cargo or transmit forces. Actin filaments and microtubules are responsible for most biological movements (for review see Pollard and Borisy, 2003; Wodarz, 2002; Pollard and Earnshaw, 2002). Both microtubules and actin filaments are dynamic structures, which allow rapid changes in the cell shape just by filament assembly and disassembly. In addition, both serve as tracks for molecular motors that are able to convert the chemical energy stored in ATP into mechanical force. Dynein and kinesin are the microtubule-based motors, while myosins are the molecular motor proteins that use actin cables as tracks (for review see Pollard and Borisy, 2003; Pollard and Earnshaw, 2002).

All myosins share a common structural organization (for review see Hasson and Mooseker, 1995; Sellers, 2000). Most myosins bear N-terminally the actin-activated ATPase. C-terminal to the ATPase is the neck region, which binds a variable number of light chains and is thought to function as a lever arm, which amplifies small conformational changes in the ATPase that occur during ATP hydrolysis. The ATPase plus the neck constitute the N-terminal motor head. C-terminal to the motor head is the class-specific tail domain.

To date, myosins are divided into 18 families. Since the type II myosins, which form the myosin filaments in muscle, were the first to be discovered, this group is called the "conventional" myosins. All others are called the "unconventional" myosins. Myosins are divided into the diverse classes based on sequence comparison of the evolutionarily conserved actin-activated ATPase (Yamashita *et al*., 2000; Sellers *et al*., 1996; Sellers and Goodson, 1995). Interestingly, the members of each myosin subtype share a common structural organization of the more divergent tail domains, suggesting that this domain specifies the cellular function of each particular myosin group (Korn, 2000; Sellers and Goodson, 1995; Sellers *et al*., 1996). Members of some specific myosin subclasses, e.g. type I myosins, are present in most eukaryotic cell types, suggesting that they fulfill evolutionarily conserved and essential functions (Hasson and Mooseker, 1995; 1996; Sellers, 2000). So far, all examined myosins, except type VI myosins, have been found to move towards the so-called barbed, or (+), end of the polar actin filaments (for review see Sellers, 2000).

The next chapters will give a general overview of type I myosins, which are the focus of this work, followed by a more detailed discussion of the role and function of these myosins in *Saccharomyces cerevisiae.*

3.1 Type I myosins

For many years, scientist believed that actin and myosin-II filaments were essential for all motility processes. In 1973, Pollard and Korn were the first to purify a monomeric protein with biochemical properties resembling myosins-II from the amoeba *Acanthamoeba castellanii* (Pollard and Korn, 1973). Although single-headed and non-filamentous, this protein was able to translocate along actin filaments. Therefore, it was subsequently termed type I myosin (myosin-I).

Type I myosins constitute a well-characterized and ubiquitous group of unconventional myosins, which bear a small, globular C-terminal tail domain (for review see Korn and Hammer, 1990; Coluccio, 1997; Sellers, 2000). The tail domains of the type I myosins contain a basic tail homology 1 (TH1) domain, which can bind acidic phospholipids and NaOH-treated, i.e. protein-stripped membranes (for review see Hasson and Mooseker, 1995; Mooseker and Cheney, 1995). The TH1 domain is therefore believed to mediate membrane-binding *in vivo*. The tail of the most "classical" type I myosins (most amoeboid and fungal type I myosins) can be further subdivided into a glycine, proline and alanine-rich tail homology 2 (GPA or TH2) domain and an Src homology 3 (SH3) domain (figure 3.1; for review see Barylko *et al*., 2000; Mooseker and Cheney, 1995). The TH2 domain is thought to contain a second ATP-independent actin binding site, since the TH2 domain of protozoal type I myosins was found to bind to actin filaments *in vitro* (Brzeska *et al*., 1988; Doberstein and Pollard, 1992; Jung and Hammer, 1994; Rosenfeld and Rener, 1994).

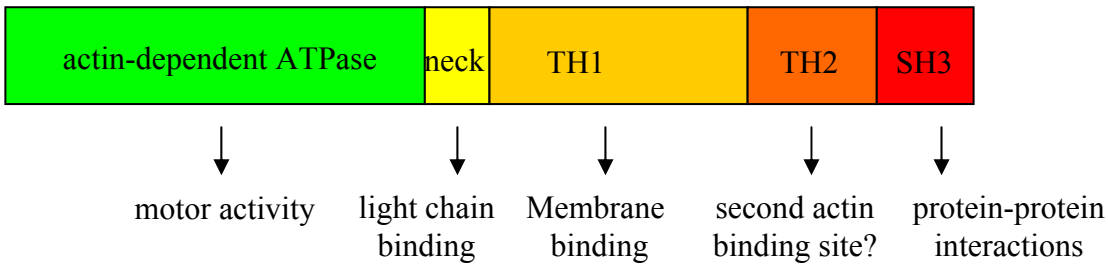

Figure 3.1: Scheme of a classical type I myosin

The drawing indicates the catalytic head (green), neck region (yellow) and the tail consisting of the membrane-binding TH1 domain (gold), the TH2 (orange) and the SH3 (red) domains of a classical type I myosin.

The presence of two actin-binding sites is thought to be responsible for the observed cross-linking of actin filaments in the presence of type I myosins (Fujisaki *et al*., 1985). SH3 domains have been found in a great variety of proteins and are implicated in protein-protein interactions through binding to proline-rich stretches (Mayer, 2001; Musacchio *et al*., 1994; Kuriyan *et al*., 1997). Fungal type I myosins contain an additional C-terminal acidic extension, which resembles the acidic domains found in members of the WASp/Scar family of proteins and which has been shown to induce actin polymerization by the Arp2/3 complex (Evangelista *et al*., 2000; Geli *et al*., 2000; Idrissi *et al*., 2002).

Other forms of the type I myosins, the "non-classical" or "short" myosins-I, lack the TH2 and SH3 domains in their tails (for review see Barylko *et al*., 2000; Coluccio, 1997). Higher eukaryotes contain two more myosin-I subclasses, which differ from the classical and short forms in their tail domains (for review see Coluccio, 1997).

Generally, most type I myosins are localized to cellular regions that undergo dynamic actin rearrangements, e.g. lamellipodia and filopodia in vertebrate and protozoal cells and actin patches in budding yeast, and they have been implicated in membrane dynamics that require actin reorganization, e.g. endocytic processes, vacuole contraction, cell locomotion and polarized cell growth (for review see Mermall *et al*., 1998; Mooseker and Cheney, 1995), as illustrated by the following examples.

The classical *Dictyostelium discoideum* type I myosins myoB, C and D are involved in actin organization (Jung and Hammer, 1990; Temesvari *et al*., 1996; Wessels *et al*., 1991) and in processes that require actin remodelling, such as motility, streaming and phagocytosis (Jung and Hammer, 1990; Jung *et al*., 1996; Titus *et al*., 1993; Wessels *et al*., 1996; Novak *et al*., 1995; Temesvari *et al*., 1996).

MyoA, the only *Aspergillus nidulans* type I myosin, is required for polarized growth and secretion (McGoldrick *et al*., 1995). A role of type I myosins in actin cytoskeleton polarization has also been demonstrated for the *Saccharomyces cerevisiae* myosins-I (Myo3p and Myo5p) (Goodson *et al*., 1996).

Myosin IC of the amoeba *Acanthamoeba castellanii* is essential for contractile vacuole function (Doberstein *et al*., 1993).

The type I myosins of *Saccharomyces*, *Acanthamoeba*, *Dictyostelium* and *Aspergillus* are required for endocytosis (Geli and Riezman, 1996; Baines *et al*., 1995; Novak *et al*., 1995; Ostap *et al*., 2003; Yamashita and May, 1998). An implication for brush border myosin-I (BBMI), which belongs to the group of short, non-classical myosins-I, in endocytosis in higher eukaryotes has also been found (Durrbach *et al*., 1996; Raposo *et al*., 1999; Durrbach *et al*.,

2000). BBMI had originally been identified as cross-linker of actin filaments to the plasma membrane in microvilli (Coluccio, 1991; Matsudaira and Burgess, 1979; Mooseker and Tilney, 1975; Mukherjee and Staehelin, 1971). In epithelial cells, BBMI has also been localized to the cytoplasmic site of Golgi-derived secretory vesicles, implying a role of type I myosins in movement of these vesicles (Fath and Burgess, 1993; Fath *et al*., 1994).

A role for classical type I myosins in organelle movement has also been assumed for *Acanthamoeba* myosins, since they co-fractionate with vesicles and since inhibition of myosin function by antibodies inhibited organelle movement *in vitro* (Adams and Pollard, 1986).

3.1.1 The myosin-I ATPase activity

As mentioned before, the myosin head domains contain the actin-activated ATPase, which can use the chemical energy of ATP to produce mechanical force. The ATP hydrolysis cycle of some myosin subclasses has been the focus of intense research. Even though not all details concerning the conformational changes that occur during ATP hydrolysis are well understood, force generation by most myosins seems to follow a common scheme: In the absence of ATP, the myosin head is tightly bound to actin. This state is called the rigor state, since tight binding of the muscular type II myosins to actin when ATP is depleted causes *rigor mortis* after death. When ATP is bound to its specific binding pocket in the head domain, myosin dissociates from the actin filament and the enzyme catalyzes the hydrolysis of ATP into ADP+P_i. This causes a conformational change of the myosin head, enabling it to rebind to actin. Release of the inorganic phosphate (P_i) allows a second conformational change, which restores the original conformation of the myosin head and thereby moves the actin filaments relative to the myosin, a process termed the power stroke. P_i release is the rate-limiting step during ATP hydrolysis by the myosin motor head. When F-actin is present, P_i dissociation is around 200 times faster than in the absence of actin. This effect is called the “actin activation of the myosin ATPase”. The advantage of this mechanism is that the motor head activity is essentially turned off when the myosin is not bound to F-actin, thus preventing unnecessary ATP consumption. After phosphate release, ADP leaves the binding pocket rapidly and the myosin head is again tightly bound to the actin filament until an ATP molecule binds to it (figure 3.2).

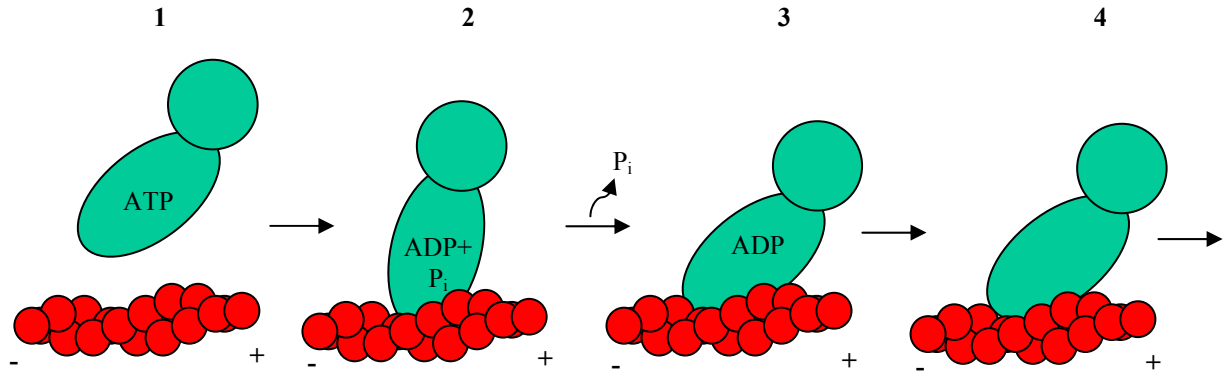

Figure 3.2: A proposed model for the myosin ATPase cycle

ATP-binding releases the myosin head from the actin filament (1). During hydrolysis of the ATP, the myosin head undergoes a conformational change that allows it to bind to the actin filament (2). Phosphate release causes a second conformational change, restoring the original, nucleotide-free, conformation (3). This conformational change moves the actin relative to the myosin (power stroke), in such a way that the myosin moves towards the barbed, (+) end of the actin filament (3). (The only known exception from this, so far, are type VI myosins, which move towards the pointed, (-) end of actin filaments.) The ADP leaves the binding pocket and the myosin stays bound to actin (4) until an ATP molecule binds again.

During the myosin ATP cycle, the motor head is tightly bound to actin only during 10-20 % of the time. As a consequence, a single myosin motor domain could not walk along an actin filament because it would quickly diffuse away from the actin filament. Thus, single headed myosins (e. g. type I myosins) or double headed myosins whose motor heads do not alternatively contact the actin filament (e. g. type II myosins) are non-processive molecular motors. Type II myosins achieve processivity by oligomerizing into thick filaments. Under these circumstances, the probability that an actin filament is bound to at least one myosin motor head approaches one and therefore, the actin filament will not diffuse away from that myosin filament. Type I myosins cannot build such long filaments, since they do not bear a long coiled-coil tail domain, which is necessary for this interaction (for review see Coluccio, 1997; Sellers, 2000). For this reason, it was suggested that processivity of myosins-I might be achieved by increasing their local concentrations, e.g. by clustering these motors at membranes (Ostap and Pollard, 1996).

3.1.1.1 Regulation of the myosin-I ATPase activity by TEDS site phosphorylation

The ATPase of a subset of type I myosins is known to be activated *in vitro* by phosphorylation of a conserved position (TEDS site) in the motor head domain (for review see Redowicz, 2001; Brzeska and Korn, 1996). This site is characterized by, and derives its name from, the presence of any one of the four amino acids threonine (T), glutamate (E), aspartate (D) or serine (S), which are found in almost any member of the myosin superfamily at this position (Bement and

Mooseker, 1995). Only myosins-I from amoeba (*Acanthamoeba castellanii* and *Dictyostelium discoideum*), fungi and yeasts (*Aspergillus nidulans*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and myosins-VI from vertebrates and *Drosophila melanogaster* carry a serine or threonine at this position (for review see Bement and Mooseker, 1995; Brzeska and Korn, 1996). *In vitro*, the ATPase of the *Acanthamoeba* and *Dictyostelium* type I myosins is activated approximately 20-50 fold by phosphorylation of this serine or threonine (Albanesi *et al*., 1983; Brzeska *et al*., 1989; Cote *et al*., 1985; Tan *et al*., 1992; Maruta and Korn, 1977; Pollard and Korn, 1973). The fact that most of the type I myosins carry a glutamate or aspartate at this position suggests that a negative charge is required for myosin-I activity (Bement and Mooseker, 1995), which makes it likely that a serine or threonine at the TEDS site will be phosphorylated for myosin function.

The TEDS site is situated in a surface loop located between the ATP binding pocket and the actin binding surface of the motor domains. Recently, it has been proposed for *Acanthamoeba* myosin IC that TEDS site phosphorylation changes the conformation of this loop and, thus, greatly enhances the phosphate release from the ATP-binding site, following the same principle that increases the ATPase activity by actin binding (Ostap *et al*., 2002).

3.1.1.2 *In vivo* significance of TEDS site phosphorylation

As mentioned before, the fact that most myosins carry a negatively charged amino acid at the TEDS site suggests that this negative charge, and therefore, in the case of some myosins-I and the myosins-VI, phosphorylation of the serine or threonine at this position, may be required for *in vivo* function. Several findings support this notion.

Immunoblot and immunoelectron microscopy analysis of the *Acanthamoeba castellanii* type I myosins IA and IB using a phospho-specific antibody revealed that 70-100 % or 10-20 %, respectively, of these proteins were phosphorylated *in vivo* (Baines *et al*., 1995). Moreover, the electron micrographs showed that phospho-myosin IA is enriched in the actin-rich cortex, especially around phagocytic cups, and that phospho-myosin IB is found to be enriched in pseudopods, filopods and pinocytic invaginations (Baines *et al*., 1995). These studies thus suggest that TEDS site phosphorylation of these myosins is required for their function(s) in phagocytosis, pinocytosis and actin reorganization at the cell cortex. The phosphorylated isoform of the third type I myosins, myosin IC, was found to be localized at the vacuolar membrane (Baines *et al*., 1995). Most interestingly, an approximately 20-fold enrichment of the phosphorylated form of this myosin was observed at the contracting vacuole, compared to the filling vacuole (Baines *et al*., 1995). This finding strongly suggests that myosin IC is

phosphorylated at the moment the vacuole contracts and that TEDS site phosphorylation of myosin IC is required for the contraction of the vacuole.

Further evidence supporting the physiological relevance of myosin-I TEDS site phosphorylation comes from genetic studies. Deletion of both *Dictyostelium discoideum* type I myosins, myoA and myoB, has been shown to impair growth, pinocytosis and actin organization (Novak *et al*., 1995). These effects are reversed by expression of wild-type myoB but not of a myoB mutant, in which the TEDS site has been mutated into an unphosphorylatable alanine (Novak and Titus, 1998).

Similarly, expression of a mutant Myo3p in *Saccharomyces cerevisiae*, in which the TEDS site serine has been mutated into alanine, did not rescue the lethality of a strain depleted of type I myosins (Wu *et al*., 1997), whereas a serine to aspartate mutant allele did, implying that the negative charge at this site, and therefore probably phosphorylation, is required for the essential function(s).

In contrast though, an unphosphorylatable (S371A) mutant of the only type I myosin in *Aspergillus nidulans*, MYOA, restored polarized hyphal growth and secretion in a MYOA deleted strain (Yamashita and May, 1998). However, TEDS site phosphorylation of MYOA also seems to influence its function *in vivo* to a certain extent, since expression of a mutant that mimics phosphorylation at this site (S371E) led to accumulation of membranes in growing hyphae, possibly due to an increase in endocytosis (Yamashita and May, 1998).

3.1.1.3 p21-activated kinases (PAKs) are TEDS site kinases

When the first *Acanthamoeba castellanii* type I myosin was purified, a co-purifying myosin I heavy chain kinase (MIHCK) activity was identified (Maruta and Korn, 1977; Pollard and Korn, 1973). Subsequently, these kinases were purified from *Acanthamoeba* and *Dictyostelium discoideum* (Hammer *et al*., 1983; Lee and Cote, 1995) and identified as members of the PAK (p21-activated kinase)/Ste20p family (Brzeska *et al*., 1997; Brzeska *et al*., 1999; Lee *et al*., 1996; Wu *et al*., 1996). PAKs constitute a family of serine/threonine kinases that are activated by the Rho-like GTPases Cdc42 and Rac, small proteins of ca. 21 kDa, which gave these kinases their name (Hall, 1994; Lim *et al*., 1996).

GTP-bound forms of Rac and Cdc42 stimulate the activity of PAKs by binding to a specific N-terminal p21-binding domain (PBD), which contains the highly conserved CRIB (Cdc42/Rac interactive binding) motif (Chung and Firtel, 1999; Lamson *et al*., 2002; Manser *et al*., 1994; Peter *et al*., 1996; Sells *et al*., 1997; for review see Aspenstrom, 1999a; Bagrodia and Cerione, 1999). Binding of the small GTPases to this site relieves the influence of an adjacent kinase autoinhibitory domain (figure 3.3; Zenke *et al*., 1999; Zhao *et al*., 1998; Tu and Wigler, 1999;

for review see Bagrodia and Cerione, 1999). Subsequently, these kinases are autophosphorylated at multiple sites, a requirement for function (Brzeska *et al*., 1999; Lee *et al*., 1998; Martin *et al*., 1995).

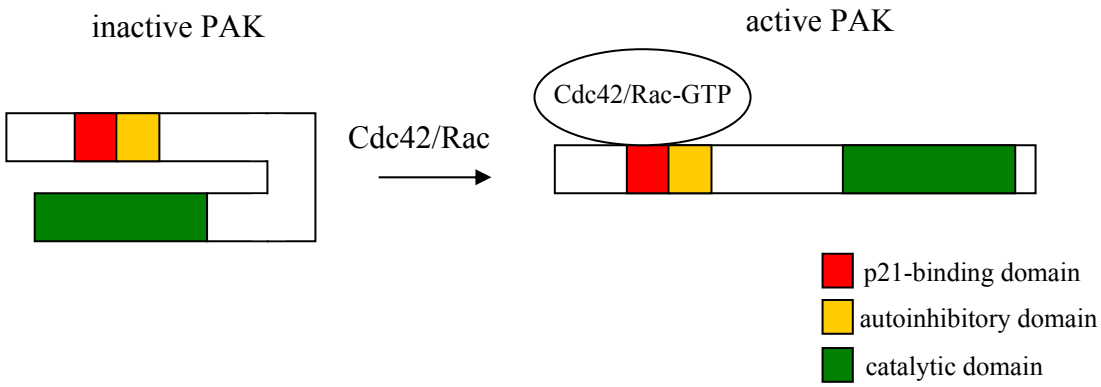

Figure 3.3: Proposed model of PAK activation

In the inactive state, the catalytic/kinase domain of p21-activated kinases is in contact with the regulatory (p21-binding and autoinhibitory) domains. Binding of Cdc42p or Rac in their GTP-bound forms leads to the release of the catalytic from the regulatory domains.

PAKs have also been shown to bind to, and be activated by, lipids, e.g. phosphatidylinositol-4,5-bisphosphate, phosphatidic acid and sphingosine (Brzeska *et al*., 1990a; Lim *et al*., 1996; Lee *et al*., 1998; Bokoch *et al*., 1998; Brzeska *et al*., 1999). It was suggested that binding of these lipids to the PAKs might also lead to a disruption of the autoinhibitory interactions (Bagrodia and Cerione, 1999).

The minimal consensus sequence for phosphorylation by the MIHCK was identified using synthetic peptides as (K,R)X_{1-3}(S,T)XY, where X is a variable residue (Brzeska *et al*., 1990b). Tertiary folding might compensate for the strict requirements of the synthetic peptides since smooth muscle myosin regulatory light chain, which does not contain the essential tyrosine, is a good substrate for the *Acanthamoeba* MIHCK (Brzeska and Korn, 1996). Moreover, Buss and co-workers suggested that PAKs phosphorylate a vertebrate type VI myosin, the only other myosin class that carries a phosphorylatable TEDS site, in a sequence that also does not completely resemble the minimal consensus sequence (Buss *et al*., 1998).

3.1.2 Some type I myosins activate the Arp2/3 complex

Actin exists in two forms, as a globular monomer, called G-actin, or polymerized in filaments, called F-actin. The filaments are double helical polymers of globular subunits arranged head-to-tail, which grow by addition of subunits at the filament ends (for review see Pollard and Borisy, 2003). The actin filament is greatly stabilized by lateral interactions between the actin

subunits of the two helical filaments, but the head to tail contacts between subunits are rather weak. Thus, actin dimers, and most likely also trimers, are highly unstable. Spontaneous *de novo* assembly of actin into filaments is therefore thermodynamically unfavourable (Pollard, 1986), allowing the cell to tightly control actin assembly at selected sites by controlling the stability of the actin dimers and trimers (the actin nucleus). Two major "nucleation bodies" that overcome the instability of actin dimers are the Arp2/3 complex and the formins. Formins trigger the formation of actin filaments that are organized in long, unbranched bundles (Evangelista *et al*., 2002; Sagot *et al*., 2002; for review see Pruyne *et al*., 2002) resembling the actin structure required for the formation of filopodia, microvilli and the yeast actin cables (DeRosier and Tilney, 2000). The Arp2/3 complex appears to bind to existing actin filaments and nucleates polymerization of actin filament branches at a 70° angle (Machesky *et al*., 1994; Mullins *et al*., 1998a; Mullins *et al*., 1998b). The resulting meshworks resembles those observed in the leading edges of migrating cells or the actin "comet tails", which some parasites use to propel themselves inside cells (Loisel *et al*., 1999; Marchand *et al*., 1995). Purified formins or Arp2/3 complex show only moderate actin nucleating activity but their activity can be increased by interaction with different activators.

The ubiquitously found Arp2/3 complex consists of seven subunits, of which two, Arp2 and Arp3, are actin-related proteins (for review see Higgs and Pollard, 2001). Arp2 and Arp3 are thought to form a stable dimer within this complex, initiating actin assembly (Kelleher *et al*., 1995). As previously mentioned, purified Arp2/3 complex displays only moderate nucleating activity (Mullins *et al*., 1998a; Welch and Mitchison, 1998; Welch *et al*., 1998). A number of cellular factors seem to locally activate this complex. The best characterized are the members of the WASp/Scar protein family (figure 3.4A; Machesky *et al*., 1999; Rohatgi *et al*., 1999; Winter *et al*., 1999; Yarar *et al*., 1999; Egile *et al*., 1999). These activators bear a C-terminal acidic domain, which binds the Arp2/3 complex (figure 3.4B; Higgs *et al*., 1999; Evangelista *et al*., 2000), and one or two WH2 (WASp homology 2) domains, which bind actin monomers (Higgs *et al*., 1999). Activation of the Arp2/3 complex requires both, the WH2 domain and the acidic domain (Higgs *et al*., 1999). Recent data indicate that additionally a poly-proline containing domain is required for efficient activation of the Arp2/3 complex (Castellano *et al*., 2001; Idrissi *et al*., 2002).

Members of the WASP family are themselves modulated, among other factors, by Cdc42p and PIP_2 (figure 3.4; for review see Higgs and Pollard, 2001; Caron, 2002) as well as by SH3 domain-containing proteins such as WIP (WASP interacting protein (figure 3.4A; for review see Caron, 2002).

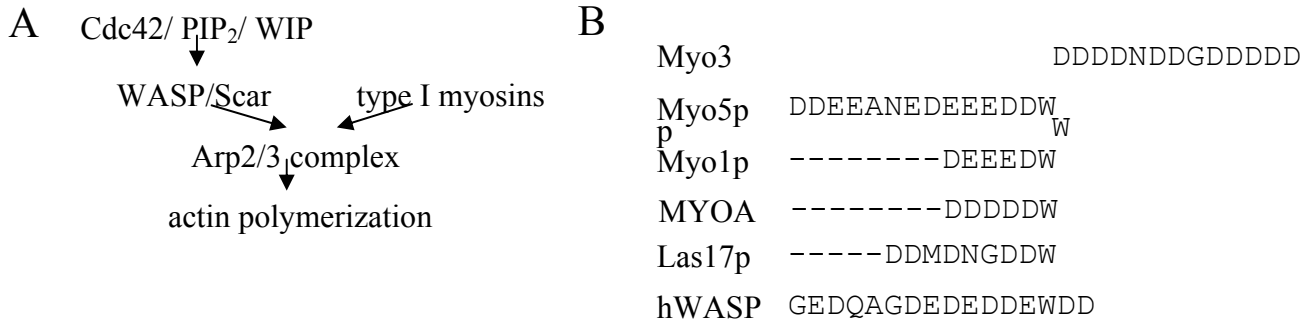

Figure 3.4: Factors that activate Arp2/3-dependent actin polymerization

(A) The evolutionarily conserved Arp2/3 complex nucleates actin polymerization. By itself, it displays only moderate nucleating activity. Factors, such as members of the WASp/Scar family of proteins can activate this complex. These activators themselves are regulated by Cdc42, PIP_2 and SH3 domain containing proteins, e.g. WIP (WASP interacting protein). Recent data indicate that fungal type I myosins are also able to activate the Arp2/3 complex. (B) Alignment of the C-terminal acidic domains of the type I myosins of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans*, Myo3p, Myo5p, Myo1p and MYOA, respectively, human WASP and the yeast WASP orthologue Las17p (according to Evangelista *et al*., 2000; Lee *et al*., 2000; Higgs and Pollard, 2001).

Recent data indicate that fungal type I myosins can induce Arp2/3-dependent actin polymerization. The *Saccharomyces cerevisiae*, *Candida albicans*, *Schizosaccharomyces pombe* and *Aspergillus nidulans* type I myosins bear acidic domains at their C-termini, which resemble the acidic domains of WASp/Scar family members (figure 3.4B; Evangelista *et al*., 2000; Jung *et al*., 2001; Lechler *et al*., 2000; Lee *et al*., 2000; Higgs and Pollard, 2001). Genetic data from the yeasts *Saccharomyces* and *Schizosaccharomyces* indicate that the acidic domains of the fungal WASP family members and the type I myosins are functionally redundant (Evangelista *et al*., 2000; Soulard *et al*., 2002; Lee *et al*., 2000). The C-terminal domains of these myosins have indeed been shown to interact with the Arp2/3 complex (Evangelista *et al*., 2000; Lee *et al*., 2000; Lechler *et al*., 2000; Geli *et al*., 2000) and to activate Arp2/3-dependent actin polymerization (Geli *et al*., 2000; Lee *et al*., 2000; Lechler *et al*., 2001; Idrissi *et al*., 2002).

Binding and activation of the actin nucleating machinery by myosins has important functional implications since polymerization of actin in the vicinity of the myosin motor head might efficiently activate the myosin ATPase and motor activity (see above).

Direct binding of the Arp2/3 complex to myosins seems to be an exception, since the acidic extensions are only found in *fungi*. However, the conservation of the myosin-I SH3 domain throughout evolution offers the possibility that adaptors that specifically bind to the myosin-I SH3 domains recruit these motors to the Arp2/3 complex. Indeed, recent data from

Dictyostelium discoideum indicate that a protein called p116 or CARMIL binds type I myosins and the Arp2/3 complex (Jung *et al*., 2001), and thus links these myosins, which lack a bona fide Arp2/3 binding motif, to the actin nucleation machinery. CARMIL is the homologue of the *Acanthamoeba* protein Acan125 (Zot *et al*., 2000; Xu *et al*., 1995; Xu *et al*., 1997) and Jung and co-workers identified homologues of these proteins in *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse and humans (Jung *et al*., 2001), suggesting evolutionary conservation.

3.2 *Saccharomyces cerevisiae* type I myosins

Budding yeast contains two type I myosins, Myo3p and Myo5p, which share 86 % identity in their motor domains and 62 % identity in their tail domains (Brown, 1997). While deletion of either gene does not result in any obvious phenotype, a double knockout of the *MYO3* and *MYO5* genes was shown to be lethal (Geli and Riezman, 1996) or very sick (Goodson *et al*., 1996), depending on the strain background. It is not known which difference in the genetic background causes the observed differences.

Both yeast myosins-I belong to the group of the "classical" type I myosins. Therefore their tail domains contain TH2 and SH3 domains, C-terminal to the positively-charged TH1 domain (figure 3.5). Additionally, these myosins contain the C-terminal acidic extension that has been shown to activate Arp2/3-dependent actin polymerization (figure 3.5; Evangelista *et al*., 2000; Geli *et al*., 2000; Idrissi *et al*., 2002; Lechler *et al*., 2000).

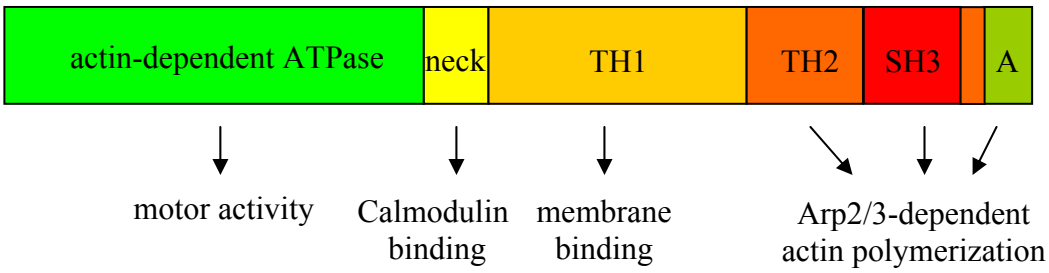

Figure 3.5: Domain structure of the yeast type I myosins.

Like type I myosins from other organisms, the yeast myosins-I contain an N-terminal motor head domain that carries the actin-dependent ATPase, followed by a neck region that binds the light chains (calmodulin) and a C-terminal tail domain. The tail consists of TH1, TH2, SH3 and acidic (A) domains. The TH1 domain is thought to mediate membrane binding. The SH3 domain divides the TH2 domain into an N-terminal and a C-terminal part. A fragment consisting of TH2, SH3 and acidic domains has been found to activate Arp2/3-dependent actin polymerization.

Despite the apparent functional redundancy of Myo3p and Myo5p (Geli and Riezman, 1996; Goodson *et al*., 1996), Myo5p appears to be the more important type I myosin in yeast. First, a *myo5Δ* strains is defective in endocytosis at 37°C, whereas a *myo3Δ* strain is not (Geli and Riezman, 1996). Second, deletion of *MYO5*, but not *MYO3*, is synthetically lethal with mutations in genes that cooperate with myosins-I *in vivo*, e.g. *VRP1*, *ARP3* (Geli *et al*., 2000). The yeast type I myosins are implicated in the uptake step of endocytosis, i.e. the formation or the primary endocytic vesicle at the plasma membrane, and in the polarization of the actin cytoskeleton (Geli and Riezman, 1996; Goodson *et al*., 1996).

Endocytosis is the process whereby cells internalize extracellular media as well as parts of their plasma membrane. Among other functions, endocytosis in yeast is used to down-regulate cell surface receptors and nutrient transporters (for review see Di Fiore and De Camilli, 2001; Geli and Riezman, 1998; Mellman, 1996; Munn, 2001).

An important finding from the studies of endocytosis in budding yeast is that this process requires actin (Kubler and Riezman, 1993). Putative roles for actin in endocytosis are (1) to provide a diffusion barrier for the endocytic machinery, (2) to help invaginating the plasma membrane in order to form a vesicle, (3) to participate in pinching the vesicle off the plasma membrane or (4) to sequester receptors into preformed endocytic sites (for review see Buss *et al*., 2001b; Geli and Riezman, 1998; Munn, 2001). The models that postulate a direct role of actin in the formation of the endocytic vesicle ((2) and (3)) imply that the actin cytoskeleton might be used to apply mechanical force on membranes, and, therefore, that an actin-dependent molecular motor might be involved in the process. Consistent with this view, it was found that the yeast type I myosins, Myo3p and Myo5p, are required for endocytosis. A temperature-sensitive *myo3Δ myo5-ts* mutant displays a strong defect in the uptake of α-factor at non-permissive temperature (Geli and Riezman, 1996). Moreover, deletion of these motor proteins in a strain background, in which this double deletion is not lethal, led to a defect in fluid phase uptake (Goodson *et al*., 1996).

Saccharomyces cerevisiae cells multiply by budding, which requires polarized growth. During the G1 phase of the cell cycle a bud site, where the daughter cell will be generated, is selected. In the subsequent S phase and the beginning of G2, the bud (daughter cell) starts being formed and growth occurs apically. At the end of G2, the growth pattern of the bud changes and it starts to expand isotropically until cytokineses occurs during mitosis (for review see Casamayor and Snyder, 2002).

The actin cytokeleton is essential for the polarized growth of a yeast cell, because it polarizes secretion and organelle inheritance (for review see Bretscher, 2003; Casamayor and Snyder,

2002; Pruyne and Bretscher, 2000a). Fluorescent F-actin staining in budding yeast shows two major structures, long cables and cortical actin patches (Adams and Pringle, 1991). Actin cables are bundles of F-actin filaments. Actin patches are motile plasma membrane invaginations that are coated with F-actin (Mulholland *et al*., 1994). During budding, actin cables align parallel to the growth axis and actin patches polarize to the growing bud (figure 3.6; for review see Pruyne and Bretscher, 2000a).

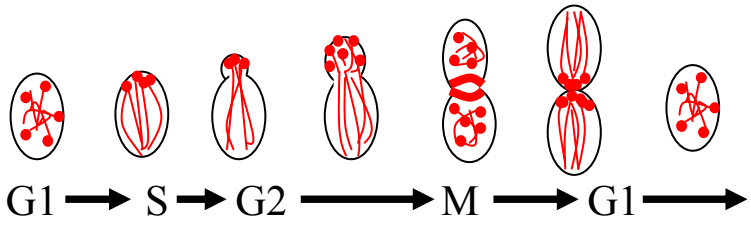

Figure 3.6: Cell polarity in budding yeast is established by the actin cytoskeleton

In a non-dividing yeast cell, actin cables and patches are randomly distributed (cell cycle phase G1). During bud emergence, actin cables align parallel to the polarity axis and actin patches are polarized to the growing bud. The actin cables guide secretory vesicles to the growing bud, thus polarizing growth (cell cycle phases S through M). During isotropic growth (G2 and M), actin patches are more randomly distributed and, especially in M phase, actin cables form a meshwork. In cytokinesis, patches accumulate at the cytokinetic ring and cables are oriented to this ring.

In a type I myosin double deletion mutant, *myo3Δ myo5Δ*, the actin patches are depolarized, distributed throughout the mother cell and the bud (Goodson *et al*., 1996), suggesting that myosins-I might be involved in actin cytoskeleton polarization in yeast.

Actin patch components, such as Cof1p, the Arp2/3 complex and the type I myosins are involved in endocytosis (Goode and Rodal, 2001; Idrissi *et al*., 2002; Geli and Riezman, 1996; Goodson *et al*., 1996). For this reason, actin patches themselves are thought to be the sites where endocytosis occurs, although this is still under discussion (Casamayor and Snyder, 2002; Pruyne and Bretscher, 2000a and b).

Since many mutants that are defective in endocytosis are also unable to polarize the actin cytoskeleton (for review see Jeng and Welch, 2001; Munn, 2001), it was suggested that actin polarization might be a prerequisite for functional endocytosis in yeast (Casamayor and Snyder, 2002; Goodson *et al*., 1996). However, not all mutants defective in actin cytoskeleton polarization are defective in endocytosis (for review see Munn, 2001), implying that endocytosis might not depend on a polarized actin cytoskeleton, but this has not yet been directly demonstrated.

3.2.1 TEDS site phosphorylation of the yeast type I myosins might be required for actin cytoskeleton polarization

Several observations suggest that Cdc42p-mediated type I myosin phosphorylation by p21-activated kinases (PAKs) might be required to regulate actin dynamics in the budding yeast *Saccharomyces cerevisiae* (figure 3.7).

Cdc42p and at least two of the three yeast members of the PAK family (Cla4p and Ste20p) seem to be required to polarize the actin cytoskeleton to the growing bud. Temperature-sensitive *cdc42* and some *cla4-ts ste20Δ* mutant strains display a depolarized actin cytoskeleton at restrictive temperature (37°C) (Benton *et al*., 1997; Cvrckova *et al*., 1995; Eby *et al*., 1998; Holly and Blumer, 1999; Peter *et al*., 1996; Lamson *et al*., 2002; for review see Pruyne and Bretscher, 2000b; Pringle *et al*., 1995) similar to the phenotype caused by deletion of the type I myosins Myo3p and Myo5p (Goodson *et al*., 1996). Further, Ste20p and Cla4p can phosphorylate the TEDS site of Myo3p *in vitro* (Wu *et al*., 1997).

In agreement with a role of Ste20p and Myo3p as downstream effectors of Cdc42p, overexpression of Ste20p overcomes the requirement for Cdc42p in actin polarization (Eby *et al*., 1998), as expression of a Myo3-S357Dp mutant mimicking the TEDS phosphorylated isoform does in a *cdc42-1* mutant strain (Lechler *et al*., 2001). Moreover, in semi-permeabilized cells, the *cdc42-1* defect in polarized actin polymerization (Lechler *et al*., 2000) can also be overcome by overexpression of Ste20p or by expression of Myo3-S357Dp (Eby *et al*., 1998; Lechler *et al*., 2000).

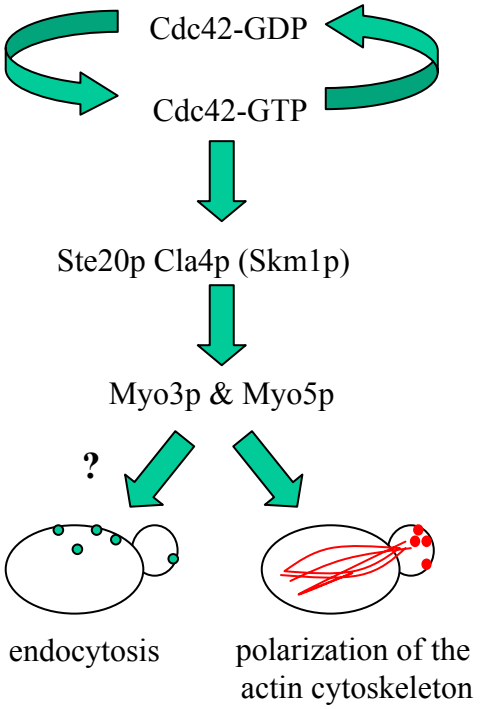

Figure 3.7: Putative signalling pathway for the activation of type I myosins for their function in actin polarization

Cdc42p, the yeast PAKs (Ste20p, Cla4p) and the type I myosins (Myo3p, Myo5p) are required for actin cytoskeleton polarization. The role of Skm1p is still unclear. PAKs phosphorylate the yeast type I myosins at the TEDS site *in vitro*. Overexpression of Ste20p and expression of Myo3-S357Dp, which mimics TEDS site phosphorylation through the charge, suppress the requirement for Cdc42p in actin polarization *in vivo* and for polarized actin polymerization *in vitro*, implying that they are downstream effectors of Cdc42p. However, *in vivo* evidence that the PAKs phosphorylate the myosins-I is missing and the involvement of PAKs in actin polarization is debated (see main text). A role of Cdc42p and PAKs in endocytosis has not yet been investigated.

Even though most results seem to point to the signalling pathway outlined in Fig. 3.7, a few results are inconsistent with the model. Intriguingly, even though overexpression of Ste20p or expression of Myo3-S357Dp suppresses the actin polarization defect of a *cdc42* mutant strain, Myo3-S357Dp was not able to suppress the lethality of a *ste20Δ cla4Δ* double knockout (Wu *et al*., 1997). Moreover, results concerning the involvement of PAKs in the actin cytoskeleton polarization are still conflicting (Eby *et al*., 1998; Holly and Blumer, 1999). In fact, recent data suggested that the observed polarization defects in temperature-sensitive *ste20Δ cla4-ts* double mutant strains might be a consequence of the 37°C heat shock applied to inactivate the mutant Cla4p protein. Weiss and co-workers have shown that inactivation of a chemical-sensitive *ste20Δ cla4-as3* double mutant caused actin depolarization at 37°C but not at 25°C, giving rise to the possibility that, in contrast to the type I myosins, PAKs might only be required for actin polarization at higher temperatures (Weiss *et al*., 2000).

In addition to the debate on whether PAKs phosphorylate the type I myosins for their function in actin polarization, it has not yet been examined whether the yeast PAKs and Cdc42p are necessary for the second function that requires type I myosin activity in yeast, the uptake step of endocytosis. Direct evidence is also missing whether myosin-I TEDS site phosphorylation is required for actin cytoskeleton polarization and endocytosis.

3.2.2 The tail of the yeast type I myosins induces Arp2/3-dependent actin polymerization

Data from our laboratory indicated that the myosin-I SH3 domain is required for the binding of actin to the myosin-I tail, which was originally solely attributed to the TH2 domain (Geli *et al*., 2000). The fact that SH3 domains mediate interactions with proteins bearing poly-proline stretches, which are not present in actin, implied that the binding of the myosin-I tail to actin might occur through adaptor proteins. Interestingly, it was found that both, Las17p (Bee1p) and Vrp1p, the yeast homologues of WASP and WIP (Ramesh *et al*., 1997; Vaduva *et al*., 1999; Winter *et al*., 1999), which activate the Arp2/3 complex in order to induce actin polymerization (Winter *et al*., 1999; Madania *et al*., 1999; Soulard *et al*., 2002), bind to the SH3 domain of the type I myosins (Evangelista *et al*., 2000; Anderson *et al*., 1998; Geli *et al*., 2000). Consistent with the idea that actin binds through adaptors to the type I myosin tail domain, it was found that the interaction of the Myo5p tail with actin was abolished in a *vrp1Δ* strain (Geli *et al*., 2000).

In addition to Las17p and Vrp1p, Arc19p and Arc40p, two subunits of the yeast Arp2/3 complex, were found to interact with the SH3 and acidic domains of Myo3p and Myo5p

(Evangelista *et al.*, 2000). In agreement with the idea that the acidic domain of the yeast type I myosins initiates actin polymerization, our laboratory demonstrated that a GST fusion protein containing the TH2, SH3 and acidic domains of Myo5p (GST-Myo5-Cp) bound to glutathione Sepharose beads can induce actin polymerization on these beads (figure 3.8; Geli *et al.*, 2000).

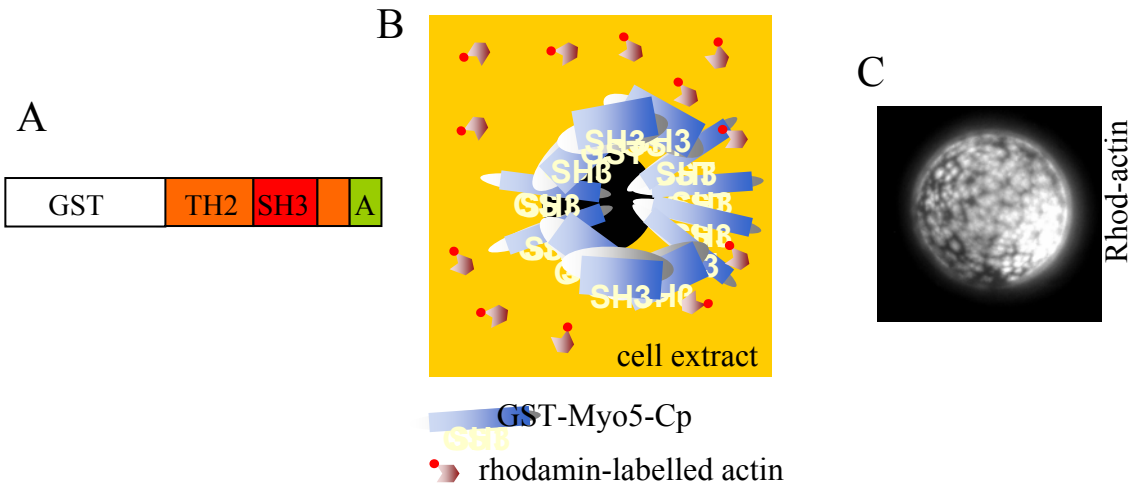

Figure 3.8: Myo5p-induced actin polymerization

(A) Schematic drawing of the structure of GST-Myo5-Cp. TH2, SH3 and A indicate the Myo5p TH2, SH3 and acidic domains, respectively. (B) The GST-Myo5-Cp protein bound to glutathione Sepharose beads is incubated with yeast extract and rhodamine-labelled actin (as indicated) in order to perform *in vitro* actin polymerization experiments. (C) Fluorescence micrographs of actin foci formed on glutathione Sepharose beads coated with the GST-Myo5-Cp protein. The beads were incubated with an extract from a wild-type yeast strain (SCMIG19) and 1µM rhodamine-labelled actin.

Further analysis of this process showed that distinct actin-containing foci appeared at the surface of these GST-Myo5-Cp-coated beads, which grew over time (Idrissi *et al.*, 2002). The Arp2/3 complex and the myosin-actin adaptor Vrp1p are required for this polymerization reaction (Geli *et al.*, 2000; Idrissi *et al.*, 2002).

As mentioned in section 3.1.2, the yeast type I myosins bear an acidic domain, similar to those in the Arp2/3 activator WASP and its yeast orthologue Las17p. Genetic data suggest a redundant role of type I myosins with Las17p in actin polymerization, since deletion of the Las17p or the myosins-I acidic domain did not show any phenotype *in vivo*, whereas double deletion of their acidic domains led to severe growth defects (Evangelista *et al.*, 2000). Although this domain binds to and activates the Arp2/3 complex, full activation of this complex requires both, a WH2 and an acidic domain (see section 3.1.2). Las17p and Vrp1p, like their mammalian counterparts WASP and WIP, carry these domains (Winter *et al.*, 1999; Lechler *et al.*, 2001; Naqvi *et al.*, 1998). Since Myo3p and Myo5p interact with these proteins through their SH3 domains, it seems likely that Vrp1p or Las17p provide the missing WH2

domain for full activation of the Arp2/3 complex by the myosin-I acidic domains. Consistent with this, Lechler and co-workers found that the WH2 domain of either Las17p or Vrp1p fused to the acidic domain of Myo3p efficiently activated the Arp2/3 complex *in vitro* (Lechler *et al*., 2001).

WASp/Scar family members are activated by Cdc42, which binds to the GTPase-binding domains (GBD) of these proteins (for review see Higgs and Pollard, 2001; Caron, 2002). In contrast, the yeast WASP orthologue Las17p does not contain a recognizable GBD domain. Nevertheless, recent data indicate that Las17p recruitment and possibly activation are mediated by activated Cdc42p (Lechler *et al*., 2001). Therefore, other proteins might transmit the signal from Cdc42p to Las17p. Soulard and co-workers found that Bzz1p, a SH3 domain-containing protein with strong similarity to the human CIP4 (Cdc42-interacting protein 4), binds to Las17p (Soulard *et al*., 2002), providing a possible mediator of Cdc42p-signalling to Las17p. Human CIP4 binds to activated Cdc42 *in vitro* and *in vivo* (Aspenstrom, 1997) and, additionally, appears to interact with the human WASP (Tian *et al*., 2000). Most interestingly, Bzz1p also binds to the C-terminal part of one of the yeast type I myosins, Myo5p and both seem to play a redundant role in actin re-polarization after salt stress (Soulard *et al*., 2002). Additionally, the finding that a *myo5Δ bzz1Δ* strain displayed salt-sensitive growth defects, while a *myo3Δ bzz1Δ* strain did not (Soulard *et al*., 2002), emphasizes the fact that Myo5p is the more important myosin-I in yeast (see above).

Some data indicate that Las17p, Vrp1p and the yeast type I myosins are found in a protein complex. Las17p, the type I myosins and Vrp1p at least partially co-localize to cortical actin patches (Anderson *et al*., 1998; Evangelista *et al*., 2000; Goodson *et al*., 1996; Lechler *et al*., 2001). Moreover, these proteins can be co-purified from enriched yeast extracts (Lechler *et al*., 2000; Soulard *et al*., 2002). However, in cell fractionation experiments, the type I myosins have not been found to be associated with a stable Las17p/Vrp1p complex, which was interpreted as an effect of dilution in this experiment (Lechler *et al*., 2001).

3.2.3 Functions of Las17p- and myosin-I-induced actin polymerization

Several findings indicate that both actin polarization and endocytosis require Arp2/3 complex-mediated actin polymerization. As mentioned before, the Rho-type GTPase Cdc42p is strictly required for the establishment of a polarized actin cytoskeleton in yeast (for review see Pruyne and Bretscher, 2000b). Vrp1p, Las17p and the yeast type I myosins are also required for actin cytoskeleton polarization (Vaduva *et al*., 1997; Zoladek *et al*., 1995; Donelly *et al*., 1993; Munn et al., 1995; Goodson *et al*., 1996; Roumanie *et al*., 2002). The polarized localization of

the Vrp1p/Las17p complex was dependent on activated Cdc42p and occurred in the absence of polarized F-actin (Lechler *et al*., 2001). After recruitment of the Vrp1p/Las17p complex, polarized F-actin-containing structures appeared, which co-localized with Las17p. These results might indicate that polarized Las17p-induced actin polymerization is required for actin polarization in yeast (Lechler *et al*., 2001). However, this complex does not seem to be sufficient for the establishment of actin polarization in yeast, since *cdc42-1* cells displayed a depolarized actin cytoskeleton, although Vrp1p/Las17p were correctly localized (Lechler *et al*., 2001). Experiments with semi-permeabilized cells indicated that not only Las17p, but also the yeast type I myosins are needed for the assembly of polarized cortical actin-containing structures (Li, 1997; Lechler *et al*., 2000). The fact that expression of a Myo3-S357Dp mutant suppressed the requirement for Cdc42p in actin polarization might indicate that the myosins-I, rather than the Vrp1p/Las17p complex, are predominantly required for Cdc42p-mediated actin polarization in yeast (Lechler *et al*., 2001). An intriguing link is provided by the finding that Myo3p and Myo5p interacted with Bni1p, one of the yeast formins (Evangelista *et al*., 2000), which seem to be required for the assembly of the actin cables (Evangelista *et al*., 2002; Sagot *et al*., 2002).

Regarding a possible function for actin assembly in endocytosis, it has been shown that temperature-sensitive *arp2* mutant strains (Idrissi *et al*., 2002; Moreau *et al*., 1996; Moreau *et al*., 1997) and mutants of the Arp2/3 activators Las17p and type I myosins, as well as Vrp1p, are defective for internalization (Geli and Riezman, 1996; Goodson *et al*., 1996; Naqvi *et al*., 1998).

4 Aim of this Work

The type I myosins of *Saccharomyces cerevisiae* exhibit two important biochemical activities. First, the ATPase activity of the motor head domain is able to use the chemical energy stored in ATP to generate mechanical force on actin. Second, the tail domain of these molecular motors is able to induce Arp2/3-dependent actin polymerization. Both activities seem to be essential for the role of type I myosins in the uptake step of endocytosis and in the actin cytoskeleton polarization. The aim of this work was to investigate the regulation of those two different myosin-I activities by phosphorylation.

It had already been known that phosphorylation at the conserved TEDS site in the yeast myosin-I motor head domain by p21-activated kinases (PAKs) activates the ATPase activity *in vitro*. However, *in vivo* evidence for the importance of this regulation for the myosin-I function in yeast was missing. Therefore, the first aim of this work was to investigate the *in vivo* significance of this phosphorylation for two particular functions of the yeast type I myosins, the uptake step of endocytosis and the polarization of the yeast actin cytoskeleton, and to characterize the signalling cascades that lead to activation of the myosin-I ATPase for each function.

Additionally, work from our laboratory had indicated that myosin-I-induced actin polymerization might be tightly regulated. Although every myosin tail is able to bind to and induce actin polymerization through the Arp2/3 complex, only a small fraction could polymerize actin in an *in vitro* assay. Therefore, the second aim of this work was to investigate whether this putative regulation is mediated by a phosphorylation/dephosporylation event and, if so, to further characterize this event.

5 Results

5.1 Analysis of Myo5p motor head phosphorylation

5.1.1 Kinases other than p21-activated kinases (PAKs) are able to phosphorylate the Myo5p TEDS site

Myo3p and Myo5p are the two highly homologous type I myosins of yeast, which share 82% similarity. Deletion of either gene does not result in any obvious phenotype for growth whereas, depending on the strain background, a double knockout is lethal or creates a very sick strain, suggesting functional redundancy (Geli and Riezman, 1996; Goodson *et al*., 1996). Wu and co-workers showed that a *myo3* mutant allele carrying a serine to alanine mutation in the TEDS site (*MYO3-S357A*) was unable to suppress the lethality of a *myo5Δ myo3Δ* double-mutation, whereas a protein bearing an aspartate at this position (*MYO3-S357D*) was restoring wild-type growth of the *myo5Δ myo3Δ* strain (Wu *et al*., 1997). This suggested that a negative charge at this position might be required for *in vivo* function and that, under physiological conditions, this charge might be provided by phosphorylation of the TEDS site serine of the wild-type protein. However, in these experiments it could not be determined if Myo3-S357Ap was expressed and if it was correctly localized.

Despite the apparent functional redundancy, Myo5p seems to play a more important role in the cell than Myo3p. At 37°C, a *myo5Δ* deletion strain is already defective in α-factor uptake, whereas a *myo3Δ* strain is not (Geli and Riezman, 1996). Moreover, deletion of *MYO5*, but not *MYO3*, causes synthetic growth defects when combined with mutations in genes that closely cooperate with the yeast type I myosins *in vivo* (Geli *et al*., 2000; Soulard *et al*., 2002; see Introduction for more detail).

Because of the functional predominance of Myo5p over Myo3p, it was possible that a substitution of serine to alanine at the Myo5p TEDS site could at least partially restore growth of the *myo5Δ myo3Δ* mutant. In this study, therefore, it was examined whether TEDS site phosphorylation plays a significant role for the *in vivo* function of Myo5p.

5.1.1.1 A negative charge at the TEDS site is required for myosin-I function

5.1.1.1.1 A TEDS site serine to alanine myo5 mutant strain is defective in endocytosis and actin cytoskeleton polarization

To examine the importance of the Myo5p TEDS site for *in vivo* function, the TEDS site serine was mutated into the non-phosphorylatable alanine and the mutant allele was integrated into the *MYO5* locus in a *myo3Δ* deletion strain (see Materials and Methods). In contrast to the findings for the *myo3-S357A myo5Δ* mutant and consistent with Myo5p playing a more important role *in vivo*, the *myo5-S357A myo3Δ* mutant was viable, although it displayed a slow-growth (3 hrs doubling time in contrast to 2 hrs for a wild-type yeast at 23°C) and temperature-sensitive lethal phenotype (figure 5.1).

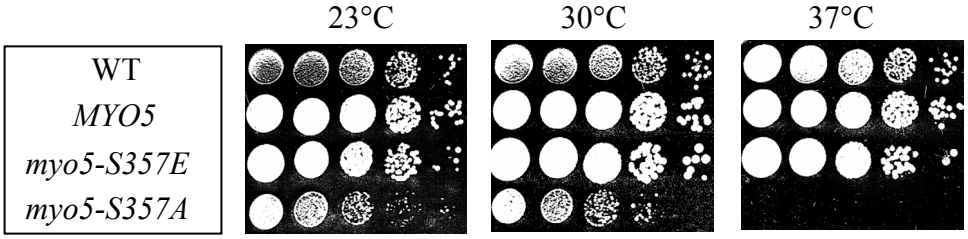

Figure 5.1: A *myo5-S357A myo3Δ* strain is viable, but temperature-sensitive for growth

Growth of wild-type (SCMIG50; WT), *MYO5 myo3Δ* (SCMIG567; *MYO5*), *myo5-S357A myo3Δ* (SCMIG568; *myo5-S357A*) and *myo5-S357E myo3Δ* (SCMIG569; *myo5-S357E*) strains was assayed at the indicated temperatures. A saturated culture was diluted to approximately 2.5 x 10^7 cells/ml and a 1 to 10 dilution series were made for each strain. 5µl of each dilution were spotted on solid rich media and the cells were grown for 2 days.

In order to mimic the active, phosphorylated state, the TEDS site serine was mutated into glutamate. This amino acid was chosen as the source for the negative charge instead of aspartate because it was found that only glutamate biochemically resembles the phosphorylated state of the *Acanthamoeba castellanii* type I myosin IC (Wang *et al*., 1998).

In contrast to the *myo5-S357A myo3Δ* mutant, the integrated *myo5-S357E myo3Δ* strain did not display a temperature-sensitive growth phenotype (figure 5.1), already indicating that a negative charge at the Myo5p TEDS site might be required for *in vivo* function. To further study this issue, two type I myosin cellular functions were examined: the uptake step of endocytosis and the actin cytoskeleton polarization (Geli and Riezman, 1996; Goodson *et al*., 1996). Endocytosis can be monitored by measuring α-factor internalization (see Materials and Methods). This assay quantitatively measures the first step of endocytosis, the endocytic uptake at the plasma membrane (Dulic *et al*., 1991). As shown in figure 5.2, a *myo5-S357A*

myo3Δ strain was not able to internalize α-factor, whereas *myo5-S357E myo3Δ* cells displayed an endocytic rate similar to wild-type myosin within the first minutes (figure 5.2A and B). Interestingly though, the *myo5-S357E myo3Δ* mutant only internalized up to 80% of the cell-bound α-factor (figure 5.2A). The reason for the earlier plateau in this mutant compared to the wild-type strain is unclear (see Discussion).

Analysis of the protein expression levels of the two mutant myosins indicated that the differences in the uptake rates were not caused by different expression levels since the amounts of Myo5-S357Ap and Myo5-S357Ep were similar (figure 5.2C).

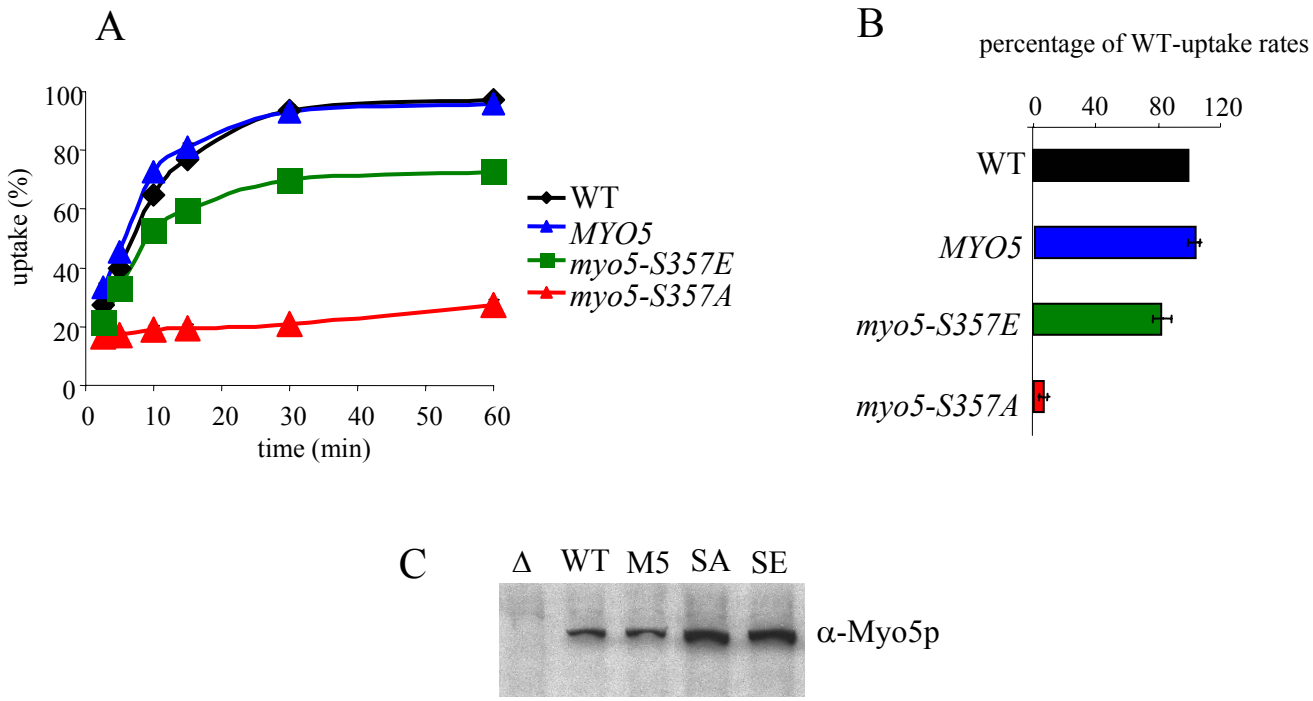

Figure 5.2: The *myo5-S357A myo3Δ* strain is unable to internalize α-factor

(A) Uptake kinetics of ^{35}S-labelled α-factor pheromone of wild-type (SCMIG50; WT), *MYO5 myo3Δ* (SCMIG567; *MYO5*), *myo5-S357A myo3Δ* (SCMIG568; *myo5-S357A*) and *myo5-S357E myo3Δ* (SCMIG569; *myo5-S357E*) strains. Cells were pulsed with ^{35}S-labelled α-factor pheromone for 45 minutes at 0°C and chased for the indicated times at 23°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points. (B) The uptake rate (% of counts internalized/minute) of the wild-type strain (SCMIG50; WT) was arbitrarily defined as 100 %, the rates of the *MYO5 myo3Δ* (SCMIG567; *MYO5*), *myo5-S357A myo3Δ* (SCMIG568; *myo5-S357A*) and *myo5-S357E myo3Δ* (SCMIG569; *myo5-S357E*) strains are calculated according to these 100 %. A mean of three different experiments is shown. (C) Equal amounts of protein from *myo5Δ* (SCMIG51; Δ), wild-type (SCMIG50; WT), *MYO5 myo3Δ* (SCMIG567; M5), *myo5-S357A myo3Δ* (SCMIG568; SA) and *myo5-S357E myo3Δ* (SCMIG569; SE) extracts were separated by SDS-PAGE and analysed by immunoblot using a Myo5p-tail-specific antibody (α-Myo5p).

Type I myosins have also been shown to be required for the polarization of actin patches to the growing bud (Goodson *et al*., 1996). As shown in figure 5.3, the phalloidin-F-actin staining of

the *myo5-S357E myo3Δ* mutant cells revealed a nearly wild-type-like polarized pattern, where the patches are mostly restricted to the growing bud. However, this pattern did not completely resemble the wild-type distribution, showing a slight depolarization (figure 5.3). In the non-phosphorylatable *myo5-S357A myo3Δ* mutant the actin cytoskeleton was completely depolarized, with cortical patches distributed throughout the mother cell and bud (figure 5.3). Cells with a depolarized actin cytoskeleton exhibit a larger size and a rounder morphology compared to wild-type yeasts, which is caused by the failure to define a polarized bud site and the resulting isotropic growth. Most of the *myo5-S357A myo3Δ* mutant cells and also some of the *myo5-S357E myo3Δ* cells displayed a big and roundish morphology (figure 5.3, morphology "*myo5-S357A*" and "*myo5-S357E*"). This observation thus corroborates the presence of a significant polarization defect in the *myo5-S357A myo3Δ* mutant and a slight polarization defect in the *myo5-S357E myo3Δ* mutant.

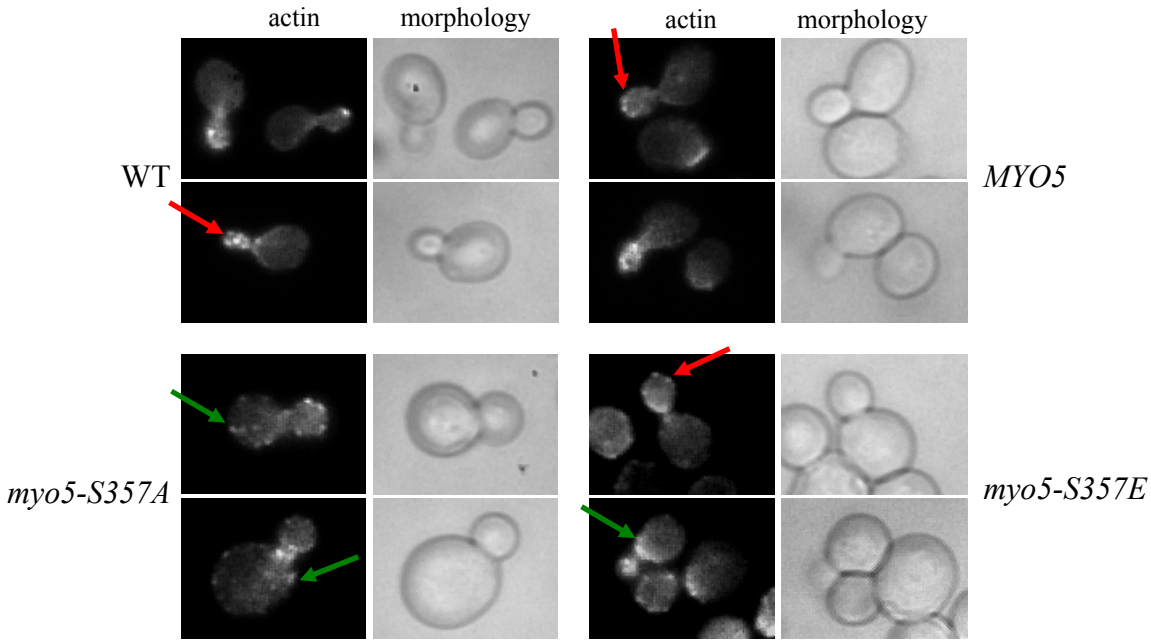

Figure 5.3: The actin cytoskeleton is depolarized in the *myo5-S357A myo3Δ* mutant

Fluorescence (actin) and phase contrast (morphology) micrographs showing wild-type (SCMIG50; WT), *MYO5 myo3Δ* (SCMIG567; *MYO5*), *myo5-S357A myo3Δ* (SCMIG568; *myo5-S357A*) and *myo5-S357E myo3Δ* (SCMIG569; *myo5-S357E*) cells stained with TRITC-phalloidin. Red arrows point to polarized actin patches, green arrows point to depolarized actin patches.

5.1.1.1.2 TEDS site phosphorylation does not seem to influence targeting of Myo5p to the plasma membrane

Based on the stability of the mutant Myo5p proteins, it seemed unlikely that the observed actin polarization and endocytic defects were caused by misfolding of the mutants (figure 5.1 and 5.2C), suggesting that phosphorylation of Myo5p was required for its function. However, it was still possible that mutation of the Myo5p TEDS site serine to alanine led to a mislocalization of the protein and that the observed defects were a consequence of the inability of the mutant protein to reach the plasma membrane. In order to test this hypothesis, immunofluorescence experiments using C-terminally Haemaglutinine (HA)-tagged proteins were performed. This tag did not disturb the myosin function in endocytosis and mutant proteins were expressed to similar levels (data not shown). In wild-type cells, Myo5p is localized in punctuate, cortical structures, which are polarized to the bud (Anderson *et al*., 1998 and figure 5.4 "*MYO5*"). The Myo5-S357Ep mutant displayed a wild-type-like Myo5p staining with most Myo5p-containing structures in the growing bud (figure 5.4). Myo5-S357Ap also seemed to be localized to the plasma membrane. Moreover, it still assembled into patch-like structures, although it showed a more depolarized distribution with evenly distributed patches in the mother cell and the bud (figure 5.4).

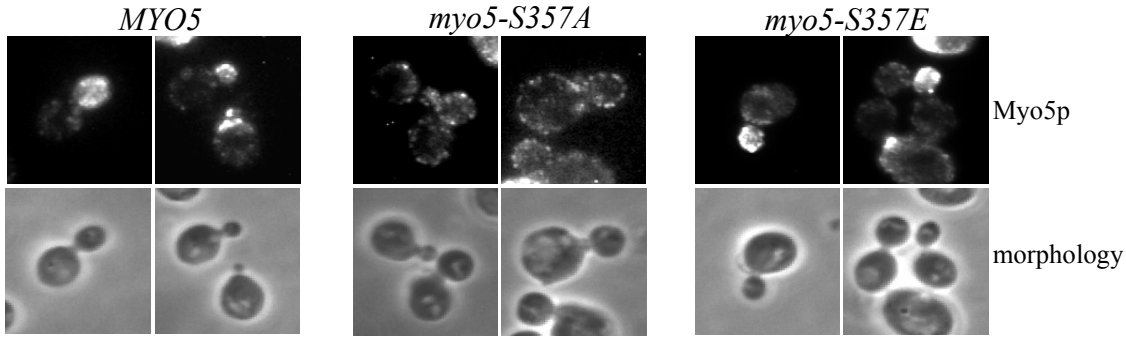

Figure 5.4: Myo5-S357Ap patches are depolarized, but seem to be at the plasma membrane

Fluorescence (Myo5p) and phase contrast (morphology) micrographs of wild-type (SCMIG50; WT), p*MYO5HA$_3$ myo3Δ* (SCMIG582; *MYO5*), p*myo5-S357AHA$_3$ myo3Δ* (SCMIG583; *myo5-S357A*) and p*myo5-S357EHA$_3$ myo3Δ* (SCMIG584; *myo5-S357E*) strains, fixed and decorated with rat α-HA antibodies and CY3-conjugated α-rat IgG antibodies.

It is likely that the Myo5-S357Ap pattern is secondary to the defect in actin polarization in these cells (compare figure 5.4 to figure 5.3), since Myo5p localization has been demonstrated to depend on actin. Anderson and co-workers demonstrated by using immunofluorescence that depolymerization of filamentous actin by Latrunculin A treatment and depolarization of actin

patches by heat shock results in depolarization of the Myo5p-containing patches (Anderson *et al*., 1998). Nevertheless, our experiments cannot rule out that TEDS site phosphorylation is required for correct targeting of Myo5p to certain membrane subdomains.

To independently confirm the recruitment of the Myo5p phosphorylation mutants to the plasma membrane, the hSos plasma membrane recruitment system was used (Aronheim and Karin, 2000). Human Sos (a guanyl nucleotide exchange factor (GEF) for Ras) can complement a temperature-sensitive mutation of its yeast homologue Cdc25p, only when hSos is correctly localized to the plasma membrane (Aronheim and Karin, 2000 and figure 5.5A). N-terminal truncation abolishes targeting of hSos to the plasma membrane preventing these cells from growing at 37°C. Fusion of this truncated hSos to a protein that is directed to the plasma membrane should rescue the growth defect (figure 5.5A).

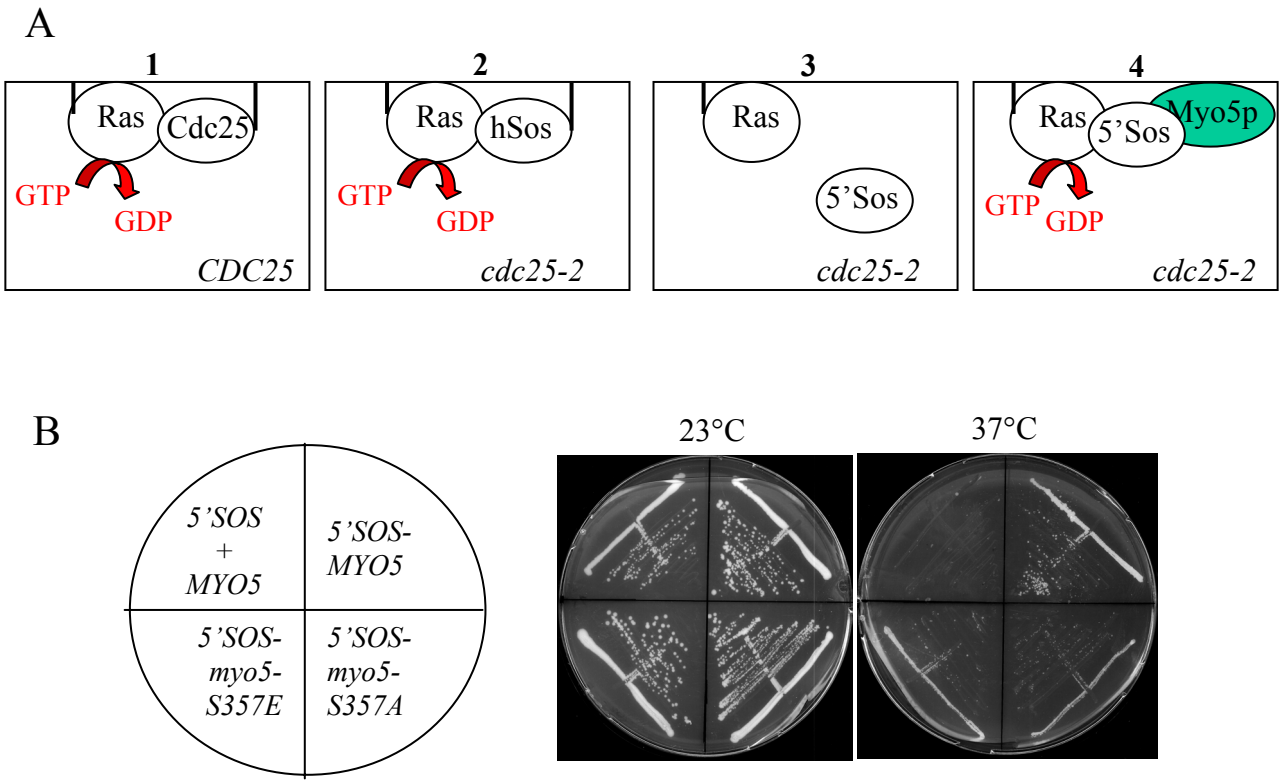

Figure 5.5: The Myo5p TEDS site mutant proteins recruit a truncated SOS to the plasma membrane

(A) Human SOS (hSOS), a guanylnucleotide exchange factor (GEF) for Ras, can substitute the function of its yeast homologue Cdc25p (*cdc25-2*) at restrictive temperature (37°C; 1 and 2). N-terminal truncation of hSOS (5'SOS) removes the membrane targeting signal, so that 5'SOS is no longer recruited to the plasma membrane (3), causing cells death at 37°C. Fusion to a protein that is recruited to the plasma membrane, e.g. Myo5p, should bring the 5'SOS to the site of function to activate Ras (4) and confer viability to the cells at restrictive temperature. (B) SCMIG271 (*cdc25-2*) was transformed with pYX*5'SOS* (*5'SOS + MYO5*), pYX*5'SOS-MYO5* (*5'SOS-MYO5*), pYX*5'SOS-myo5-S357A* (*5'SOS-myo5-S357A*) or pYX*5'SOS-myo5-S357E* (*5'SOS-myo5-S357E*), cells were streaked on minimal media and allowed to grow at the indicated temperatures for 4 days.

As shown in figure 5.5B, a fusion of the N-terminally truncated hSos to wild-type Myo5p partially restored growth at 37°C, consistent with Myo5p plasma membrane localization. Fusions of either Myo5-S357Ap or Myo5-S357Ep to the N-terminal truncated hSos were just as efficient as wild-type Myo5p in restoring cell growth indicating that the mutant Myo5p proteins were able to recruit the fusion proteins to the plasma membrane (figure 5.5B).
The results from the SOS recruitment system are thus consistent with the immunofluorescence data, both indicating that recruitment of Myo5p to the plasma membrane does not depend on TEDS site phosphorylation.

5.1.1.1.3 Other membrane traffic events are not affected in myosin-I TEDS site mutants

Since both myosin-I mutants were constitutive, it could not be excluded that the observed defects in the *myo5-S357A myo3Δ* mutant were secondarily caused by the strain sickness. To rule out this possibility, the biosynthetic traffic of carboxypeptidase Y (CPY) from the ER to the vacuole was examined as an example for a myosin-I-independent process (Geli and Riezman, 1996). Wild-type kinetics of CPY maturation requires intact protein synthesis and translocation into the ER, proper membrane traffic through the Golgi, where the CPY is glycosylated, and transport across the endosomal compartments into the vacuole, where CPY is proteolytically cleaved (Stevens *et al*., 1982). These trafficking steps create the CPY protein forms p1 in the ER, p2 in the Golgi, and the mature (m) form in the vacuole. As shown in figure 5.6, maturation and transport of CPY did not display a defect in any of the type I myosin mutants, suggesting that the defects observed in endocytosis and actin cytoskeleton polarization were specific.

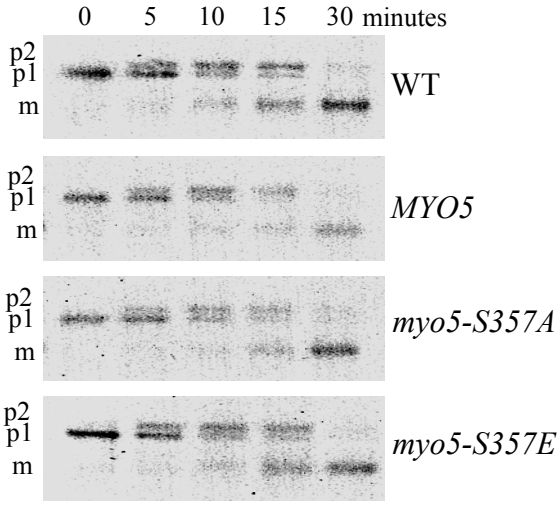

Figure 5.6: CPY maturation is unaffected by mutation of the Myo5p TEDS site

Wild-type (SCMIG50; WT), *MYO5 myo3Δ* (SCMIG567; *MYO5*), *myo5-S357A myo3Δ* (SCMIG568; *myo5-S357A*) and *myo5-S357E myo3Δ* (SCMIG569; *myo5-S357E*) cells were pulsed with ^{35}S-methionine and ^{35}S-cysteine for 5 minutes and chased for the indicated times. CPY was immunoprecipitated and analysed by autoradiography; p1, endoplasmic reticulum form; p2, Golgi form; m, mature vacuolar form.

Taken together, these results indicate that a negative charge at the TEDS site position of the yeast Myo5p, and thus presumably phosphorylation of the TEDS site serine, is important for Myo5p function *in vivo*.

5.1.1.2 The yeast p21-activated kinases (PAKs) and Cdc42p are not required for the uptake step of endocytosis

5.1.1.2.1 A temperature-sensitive PAK mutant strain displays normal endocytic uptake kinetics

Several findings indicate that Cdc42p-activated PAKs are able to phosphorylate the yeast type I myosin TEDS site and therefore activate these molecular motors for their function in actin cytoskeleton polarization (figure 5.7; for more details see Introduction).

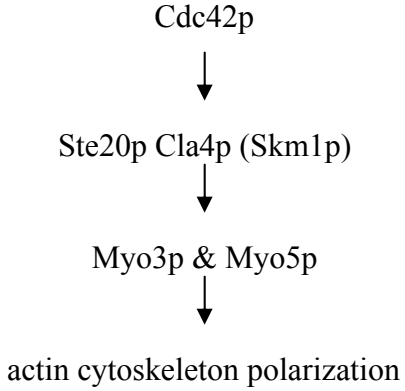

Figure 5.7: A proposed signalling pathway leading to actin cytoskeleton polarization in yeast

Cdc42p, the p21-activated (PAK) kinases Ste20p and Cla4p and the type I myosins Myo3p and Myo5p are required for actin cytoskeleton polarization. The function of Skm1p is not yet clear. Overexpression of Ste20p and an activated Myo3p TEDS site mutant (*myo3-S357D*) were able to overcome the need for a functional Cdc42p in actin polarization. Moreover, PAKs have been shown to phosphorylate the type I myosin TEDS site *in vitro*. These data thus suggest a pathway as schematically shown here.

Since the data presented in the last sections indicated that TEDS site phosphorylation is required for both type I myosin functions, actin cytoskeleton polarization and endocytosis, it was examined whether PAKs are required for endocytosis.

Cells deleted for *CLA4* or *STE20* are viable (Cvrckova *et al*., 1995; Leberer *et al*., 1992), but deletion of both genes causes synthetic lethality suggesting functional redundancy (Cvrckova *et al*., 1995). In order to create a conditional triple PAK mutant strain to test the putative role of these kinases in endocytosis, a temperature-sensitive *ste20Δ skm1Δ cla4-ts* mutant was created. For this reason, histidine 685 in *CLA4* was substituted by tyrosin (H685Y) in a *ste20Δ skm1Δ* knockout background (see Materials and Methods). An analogous mutation in the yeast casein kinase I *YCK2* has been shown to cause temperature-sensitive lethality (Robinson *et al*., 1993).

As expected, the *ste20Δ skm1Δ cla4-H685Y* strain was temperature-sensitive for growth (figure 5.8A). At permissive temperature, some of the mutant cells displayed elongated buds

similar to those described in the *ste20Δ cla4-75* mutant (figure 5.8B; Cvrckova *et al*., 1995). Upon shift to 37°C, the *ste20Δ skm1Δ cla4-H685Y* cells adopted a round morphology (figure 5.8B). As described above, this phenotype is a consequence of a failure to define a polarized bud site and the resulting isotropic growth. The fact that a similar phenotype has been described in other *ste20Δ cla4* mutants at this temperature (Holly and Blumer, 1999; Weiss *et al*., 2000) combined with the growth defect thus provide strong evidence for a significant loss of activity of the Cla4-H685Yp kinase at 37°C.

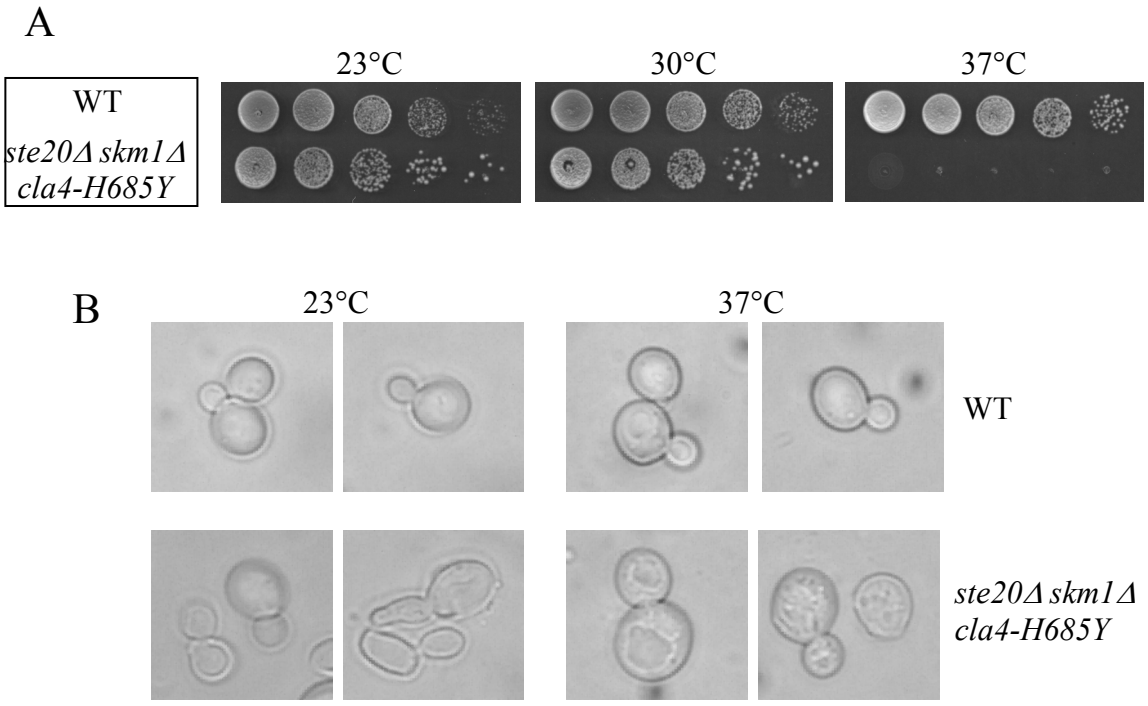

Figure 5.8: Temperature-sensitivity and morphology of the *ste20Δ skm1Δ cla4-H685Y* mutant

(A) Growth of the wild-type (SCMIG50; WT) or the *ste20Δ skm1Δ cla4-H685Y* mutant (SCMIG574; *ste20Δ skm1Δ cla4-H685Y*) strain at the indicated temperatures. A saturated culture was diluted to approximately 2.5 x 10^7 cells/ml and a 1 to 10 dilution series was made for each strain. 5 µl of each dilution was spotted on solid rich media and the cells were grown for 2 days. (B) Phase contrast micrographs of the wild-type (SCMIG50; WT) or the *ste20Δ skm1Δ cla4-H685Y* mutant (SCMIG574; *ste20Δ skm1Δ cla4-H685Y*) strains after incubation for 30 minutes at the indicated temperatures.

To investigate whether PAKs are required for the uptake step of endocytosis, the α-factor internalization kinetics of *ste20Δ skm1Δ cla4-H685Y* mutant cells were investigated upon shift to the restrictive temperature (37°C). Surprisingly, inactivation of PAK function by pre-incubation of the temperature-sensitive mutant at 37°C for 5, 15 or even 30 minutes did not lead to any defect in α-factor internalization (figure 5.9).

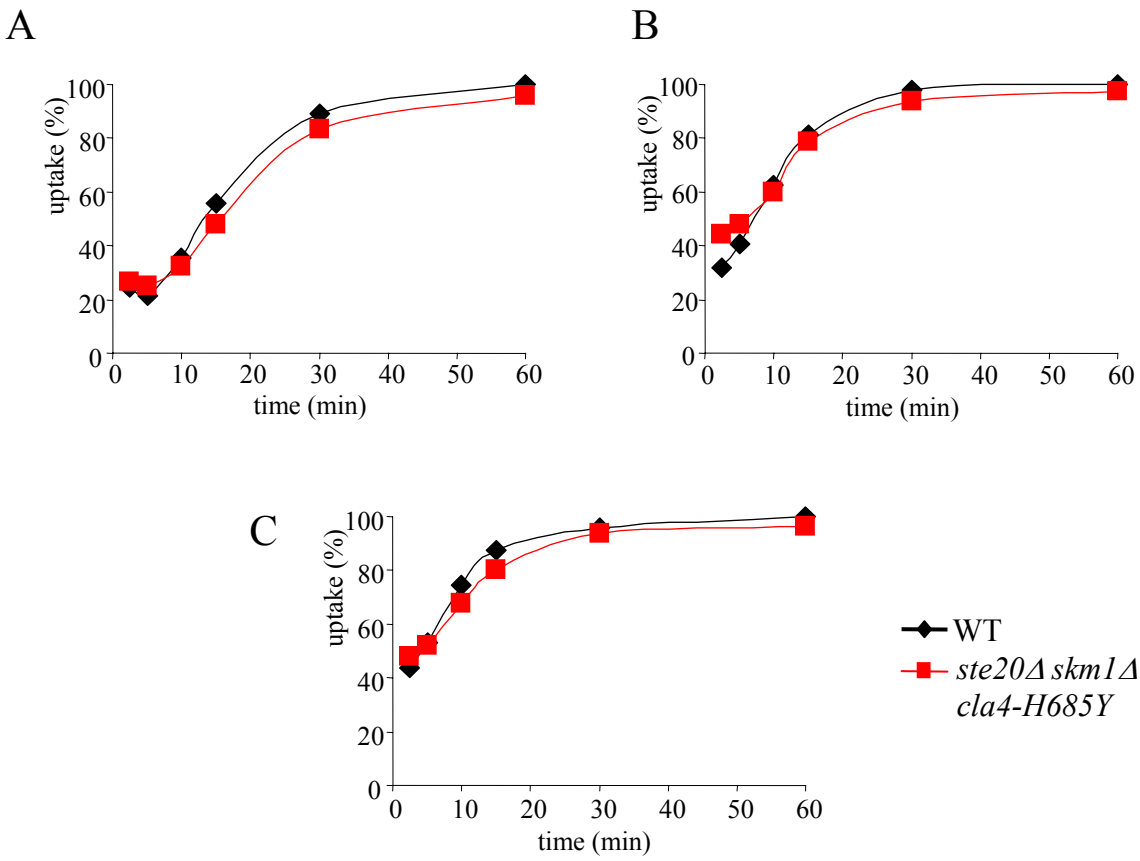

Figure 5.9: α-factor uptake kinetics are not affected in the *ste20Δ skm1Δ cla4-H685Y* temperature-sensitive mutant

Uptake kinetics of ^{35}S-labelled α-factor pheromone of a wild-type (SCMIG50; WT) and the *ste20Δ skm1Δ cla4-H685Y* mutant (SCMIG574; *ste20Δ skm1Δ cla4-H685Y*) strain. Cells were preincubated at 37°C for 5 (A), 15 (B) or 30 minutes (C), ^{35}S-labelled α-factor pheromone was added and internalization was allowed for the indicated times at 37°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points.

5.1.1.2.2 Chemical inactivation of the yeast PAKs does not affect endocytosis

The above-described experiments could not rule out that a residual PAK activity in the *ste20Δ skm1Δ cla4-H685Y* mutant was sufficient to sustain myosin-I function *in vivo*. To address this question it was necessary to demonstrate that under the exact conditions used to assay endocytosis in a PAK mutant, the actin cytoskeleton polarization was affected, indicating that the PAK signalling has completely been interrupted. Unfortunately, the temperature shift that is required to inactivate the temperature sensitive PAK mutant causes a rapid, transient and *RHO1*-dependent depolarization of the actin cytoskeleton even in wild-type cells (Delley and Hall, 1999). This fact did not allow to monitor the effect of PAK inactivation on actin polarization in the temperature-sensitive mutant under the conditions used to investigate endocytosis. To overcome this problem, a chemical-sensitive *CLA4* allele was used: *cla4-as3* (Weiss *et al*., 2000).

Shokat and co-workers were the first to engineer an adenosine-related [3,4-*d*]pyrimidine analogue that specifically binds to a kinase with a mutationally enlarged ATP binding pocket (for review see Bishop *et al*., 2001). Weiss and co-workers constructed a Cla4p mutant, Cla4-as3p, which can be inhibited specifically and efficiently by the membrane-permeable pyrimidine analog 1NM-PP1 (4-amino-1-tert-butyl-3-(1-naphtylmethyl) pyrazolo [3,4-d] pyrimidine; Weiss *et al*., 2000). This kind of conditional mutant allowed to monitor the effect of PAK inactivation on the actin cytoskeleton polarization and endocytosis under identical restrictive conditions without the interference of the heat shock.

It was previously shown that the *in vivo* activity of Cla4-as3p is inhibited by incubation for 1 hour with 25 μM 1NM-PP1 (Weiss *et al*., 2000). Under these conditions, endocytosis was not affected in a *ste20Δ skm1Δ cla4-as3* mutant strain (data not shown). Increasing the amount of inhibitor to 100 μM 1NM-PP1 did also not alter the internalization kinetics (figure 5.10A).

Under the same conditions, the actin cytoskeleton displayed a completely depolarized actin cytoskeleton as evidenced by phalloidin-F-actin staining in the *ste20Δ skm1Δ cla4-as3* mutant strain (figure 5.10B), indicating that Cla4p activity, and, therefore, PAK activity, was indeed abrogated in this mutant strain.

These data provide the first evidence that PAKs are required for actin polarization under non-heat shock conditions. As mentioned in the introduction, it was previously shown that Myo3-S357Dp was unable to restore growth of a *ste20Δ cla4Δ* double mutant indicating that myosins-I were not the essential PAK targets. Consistent with this result, Myo5-S357Ep did not suppress the actin polarization defect of the triple PAK mutant under restrictive conditions (figure 5.10C).

These data thus confirmed our findings with the temperature-sensitive mutant and supported the view that PAKs are indeed not required for endocytic internalization in yeast. Additionally, the presented data clearly demonstrated that the uptake step of endocytosis did not depend on the polarization of the actin cytoskeleton (compare figure 5.10A and B).

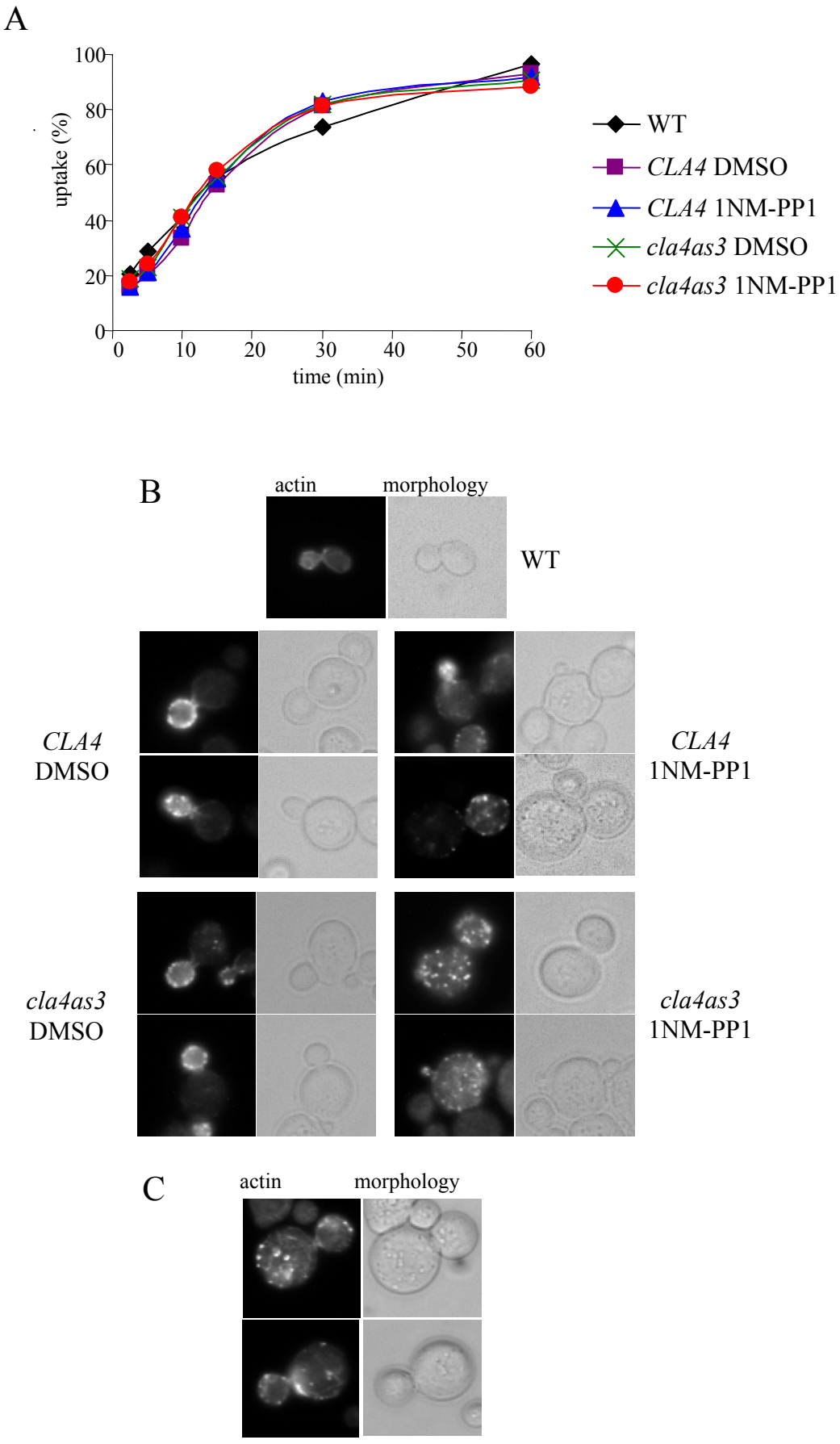

Figure 5.10: Endocytosis is unaffected in a chemical-sensitive PAK mutant strain, which displays a completely depolarized actin cytoskeleton under the same conditions

(A) Uptake kinetics of ^{35}S-labelled α-factor pheromone of wild-type (SCMIG50; WT), *ste20Δ skm1Δ CLA4* (SCMIG588; *CLA4*) or *ste20Δ skm1Δ cla4as3* (SCMIG586; *cla4as3*) strains. Cells were preincubated with 100 µM Cla4-as3p inhibitor 1NM-PP1 (1NM-PP1) or the adequate amount of solvent (DMSO) for 1 hour, ^{35}S-labelled α-factor pheromone was added and the internalization was allowed for the indicated times at 23°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points. (B) Fluorescence (actin) and phase contrast (morphology) micrographs showing wild-type (SCMIG50; WT), *ste20Δ skm1Δ CLA4* (SCMIG588; *CLA4*) or *ste20Δ skm1Δ cla4as3* (SCMIG586; *cla4as3*) cells stained with TRITC-phalloidin. Cells were preincubated with 100 µM Cla4-as3p inhibitor 1NM-PP1 (1NM-PP1) or the adequate amount of solvent (DMSO) for 1 hour. (C) Fluorescence (actin) and phase contrast (morphology) micrographs showing *ste20Δ skm1Δ cla4as3* (SCMIG586) cells, which are transformed with p*myo5-S357E*, stained with TRITC-phalloidin. Cells were preincubated with 100 µM Cla4-as3p inhibitor 1NM-PP1 for 1 hour.

5.1.1.2.3 Cdc42p mutant strains are able to internalize α-factor with wild-type kinetics

The previous results strongly suggested that PAK activity is not required for endocytosis. In order to confirm these data, it was tested whether the PAK upstream factor Cdc42p is also dispensable for this process. For this purpose, temperature-sensitive *cdc42* mutant alleles, which were previously shown to specifically disrupt PAK signalling, were tested for their ability to internalize α-factor.

A

mutant allele	growth phenotype suppressed by
cdc42-1	Ste20p
cdc42-118	Ste20p
cdc42-123	Ste20p
cdc42-129	Cla4p/Skm1p

B

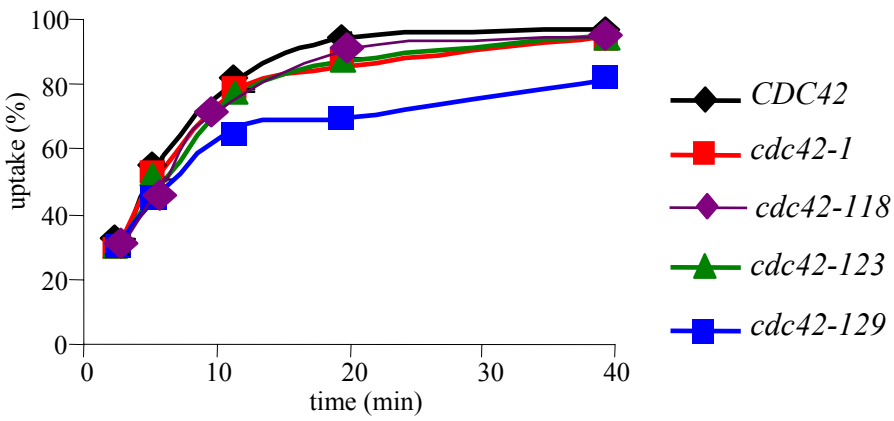

Figure 5.11: Cdc42p does not seem to be required for endocytosis

(A) Allele-specific interaction of *cdc42* temperature-sensitive mutants and PAKs (Kozminski *et al.*, 2000). (B) Uptake kinetics of ^{35}S-labelled α-factor pheromone of wild-type (SCMIG618; *CDC42*), *cdc42-1* (SCMIG619; *cdc42-1*), *cdc42-118* (SCMIG625; *cdc42-118*), *cdc42-123* (SCMIG623; *cdc42-123*) and *cdc42-129* (SCMIG624; *cdc42-129*) strains. Cells were preincubated at 37°C for 30 minutes, ^{35}S-labelled α-factor pheromone was added and the internalization was allowed for the indicated times at 37°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points.

The temperature sensitive growth of *cdc42-1*, *cdc42-118* and *cdc42-123* was specifically suppressed by overexpression of Ste20p, whereas that of the *cdc42-129* mutant could be rescued by overexpression of Skm1p or Cla4p (figure 5.11A; Kozminski *et al*., 2000).
Consistent with the results using the PAK mutants, the *cdc42-1*, *cdc42-118, cdc42-123* and *cdc42-129* cells displayed nearly wild-type α-factor uptake kinetics after 5 min, 15 min and even after 30 min preincubation at restrictive temperature (figure 5.11B and data not shown).

5.1.1.3 Only a small fraction of the Myo5p TEDS site seems to be phosphorylated *in vivo*

Since the data presented in the previous sections indicated that the yeast type I myosin TEDS site is phosphorylated by kinases other than PAKs, inactivation of these kinases should not lead to a complete loss of Myo5p phosphorylation *in vivo*. To investigate this matter, an *in vivo* phosphorylation assay was developed. Cells deleted for *MYO5* (*myo5Δ*) carrying either *MYO5-HA$_3$* or *myo5-S357A-HA$_3$* were radiolabelled, lysed and Myo5p or Myo5-S357Ap were immunoprecipitated by a Myo5-tail-specific antibody (for more detail see Materials and Methods). As described before, the HA tag did not disturb the functionality of Myo5p (data not shown).
Surprisingly, Myo5-S357Ap was labelled to the same extent as the wild-type Myo5p (figure 5.12B, lower panel), indicating that additional phosphorylation sites exist in the Myo5p protein. Interestingly, the lack of a significant decrease in phosphorylation signal in the mutant protein suggested that only a small fraction of Myo5p is phosphorylated at the TEDS site *in vivo* (figure 5.12B, lower panel). Additionally, no phosphorylation signal could be detected on a Myo5p truncation bearing only the head domain (figure 5.12C). This result might either reinforce that only a small fraction of Myo5p is phosphorylated at the TEDS site or it might indicate that recruitment of Myo5p to the plasma membrane is required for TEDS site phosphorylation, since the Myo5p head domain is not sufficient to target the protein to the plasma membrane (H. Groetsch, personal communication).

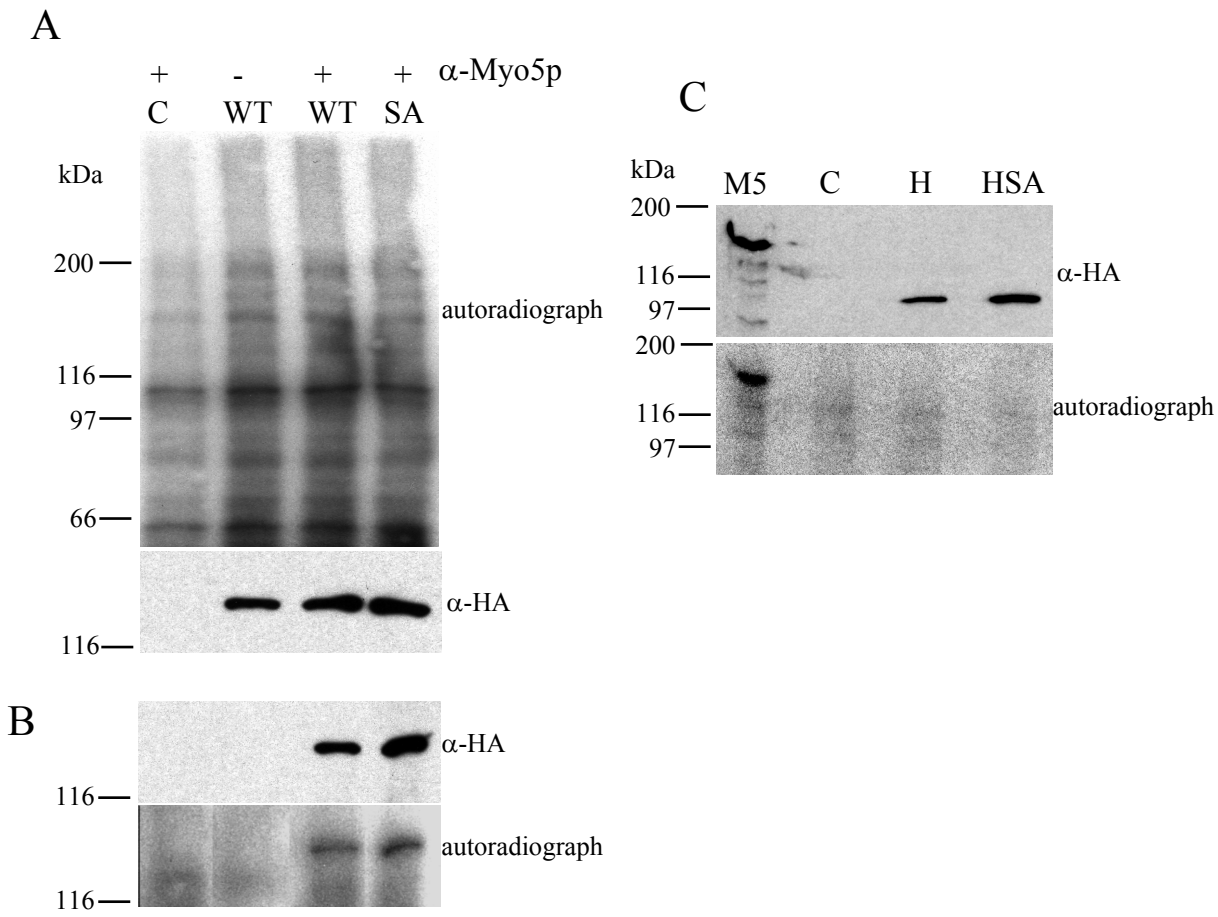

Figure 5.12: A little fraction of Myo5p seems to be phosphorylated at the TEDS site *in vivo*

(A) Cells deleted for *MYO5* carrying either an empty plasmid (YCplac33; C) or the same plasmid carrying the *MYO5HA$_3$* (p33*MYO5HA$_3$*; M5) or the *myo5-S357AHA$_3$* (p33*myo5-S357A HA$_3$*; SA) genes, were radiolabelled *in vivo* with $^{32}PO_4^{3-}$ for 30 minutes. Subsequently, cells were lysed and 1/10th of the totals were separated by SDS-PAGE and analysed by autoradiography (upper panel) and immunoblot (lower panel) using a rat α-HA antibody (α–HA). (B) Cells deleted for *MYO5* carrying either an empty plasmid (YCplac33; C) or the same plasmid carrying the *MYO5HA$_3$* (p33*MYO5HA$_3$*; M5) or the *myo5-S357AHA$_3$* (p33*myo5-S357A HA$_3$*; SA) genes, were radiolabelled *in vivo* with $^{32}PO_4^{3-}$ for 30 minutes, lysed and protA-Sepharose beads were added either alone (-) or together with a Myo5p tail specific antibody (+) (see (A)). Half of the immunoprecipitated proteins were separated by SDS-PAGE and analysed by immunoblot (upper panel) using a rat anti-HA antibody (α–HA) or by autoradiography (lower panel). (C) Cells deleted for *MYO5* carrying the plasmid-based full-length *ProtAMYO5HA$_3$* (p*ProtAMYO5-HA$_3$*, M5), *MYO5HA$_3$* (p33*MYO5HA$_3$*, C) or the ProtA-Myo5p head domain fusions *ProtAMYO5* (aa2-700) (p33*ProtA-myo5*-H-*HA$_3$*, H) or *ProtAmyo5-S357A* (aa2-700) (p33*ProtA-myo5-S357A*-H-*HA$_3$*, HSA), were radiolabelled *in vivo* with $^{32}PO_4^{3-}$ for 30 minutes, lysed and IgG beads were added. Half of the immunoprecipitated proteins were separated by SDS-PAGE and analysed by immunoblot (upper panel) using a rat anti-HA antibody (α–HA) or by autoradiography (lower panel).

5.1.1.4 The yeast kinases Pkh1p and Pkh2p may be myosin-I upstream factors

5.1.1.4.1 An in vitro *phosphorylation screen identified kinases other than PAKs that are able to phosphorylate the Myo5p TEDS site*

The results shown above strongly suggested that TEDS site phosphorylation is required for Myo5p function in endocytosis whereas the yeast PAKs are not necessary for this process. Thus, kinases other than Ste20p, Cla4p and Skm1p are likely to phosphorylate the TEDS site of the yeast type I myosins in order to activate them for their function in endocytosis. To identify this (these) kinase(s), an *in vitro* phosphorylation screen was performed in co-laboration with the laboratory of Dr. Michael Snyder (Zhu *et al*., 2000). For this purpose, a glutathione-S-transferase (GST) fusion protein carrying amino acids 322 to 391 of the Myo5p head domain, which contains the TEDS phosphorylation site (amino acid 357), was expressed and purified from *Escherichia coli* (figure 5.13A and B). Purified proteins (GST-Myo5-TEDSp and GST as control) were crosslinked to multi-well plates and independently incubated in the presence of 119 protein kinases purified from yeast and γ^{33}P-ATP (Zhu *et al*., 2000). Analysis of two independent experiments showed that 16 out of the 119 purified yeast kinases exhibited a significantly higher phosphorylation signal in the GST-*myo5*-TEDS chip compared to the GST control (figure 5.13C).

Strikingly, the PAKs Ste20p, Cla4p and Skm1p were not able to phosphorylate the Myo5p TEDS site in this biochemical screen (figure 5.13C). This might be explained by the absence of Cdc42p in the assay, which is required to activate these kinases (Peter *et al*., 1996; Benton *et al*., 1997; Cvrckova *et al*., 1995; Leberer *et al*., 1992).

Some of the identified kinases are localized in the nucleus and/or have been found to be involved in nuclear processes (Kin1p, Ksp1p, Sch9p, Ptk2p, Ssn3p, Pho85p), are required for cell cycle regulation (Tpk1p, Swe1p), conjugation during cellular fusion (Prr1p, Cmk2p) or unfolded protein response (Ire1p, Cmk2p), making all of them unlikely activators of type I myosins for their function in endocytosis. In contrast, Mkk1p and Mkk2p were found to be required for actin cytoskeleton organization and cortical actin patch assembly (Harrison *et al*., 2001; Loewith *et al*., 2002). However, an *mkk1Δ mkk2Δ* double mutant was not defective in α-factor uptake (data not shown). Likewise, Ypl141Cp, a protein kinase with unknown function, was also not required for endocytosis (data not shown).

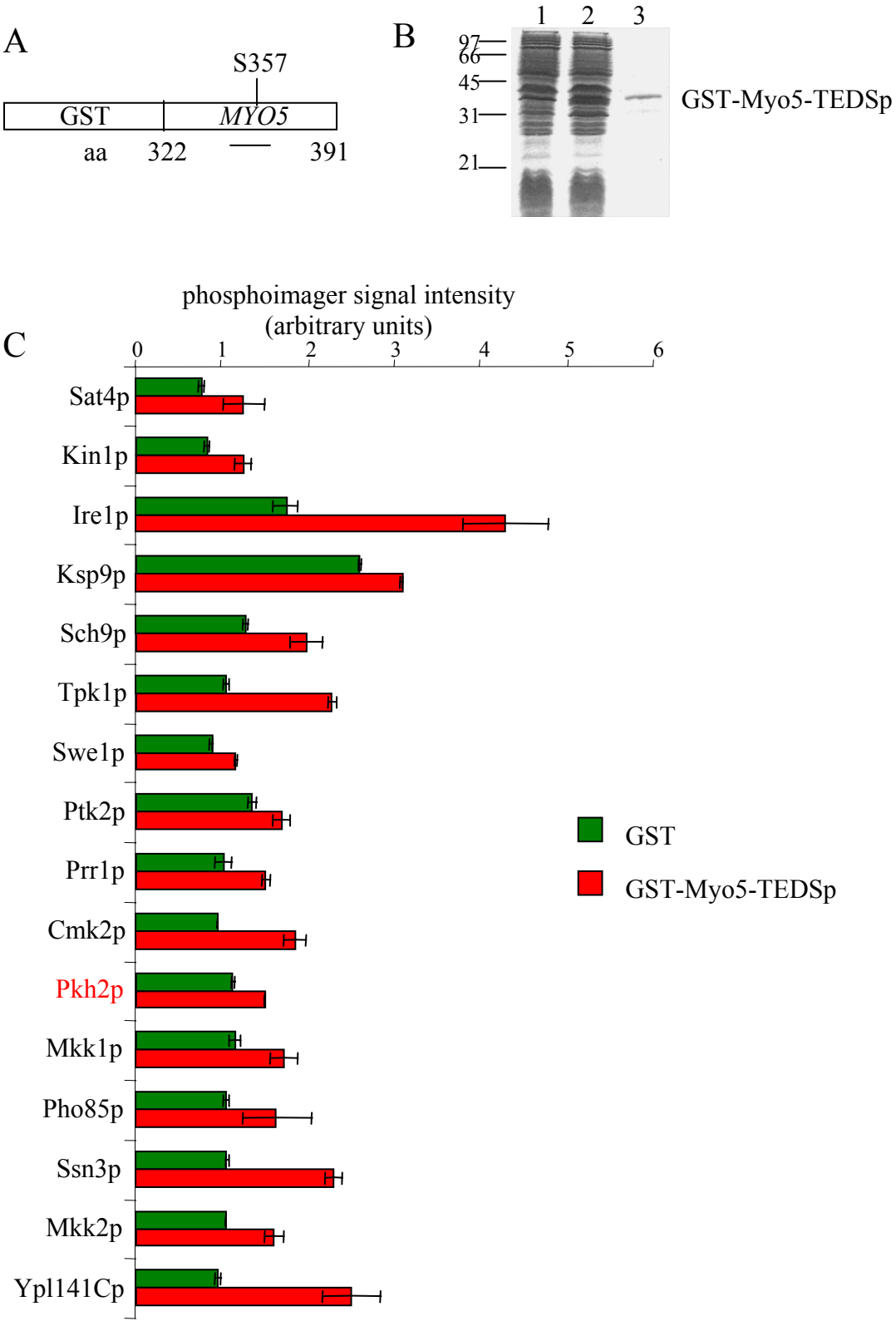

Figure 5.13: *In vitro* screen for Myo5p TEDS site kinases

(A) Schematic drawing of the GST-Myo5-TEDSp fusion protein. Amino acids 322-391 of the Myo5p protein, containing the TEDS site serine at position 357, were cloned in frame behind the GST. (B) Cell extracts of *E.coli* BL21 carrying plasmid pGST-*myo5*-TEDS before (1) and after induction (2) with IPTG. Lane 3 shows the amount of purified protein (see Materials and Methods) from approximately 6.7 ml of *E.coli* culture. (C) 119 yeast kinases were tested for their ability to phosphorylate GST-Myo5-TEDSp *in vitro*. The bars indicate the average radioactive signal (phosphoimager arbitrary units) associated to GST-*myo5*-TEDSp or GST incubated with the indicated yeast kinases, which were able to significantly phosphorylate the TEDS site fragment *in vitro*.

Interestingly though, one of the identified kinases, Pkh2p and its homologue Pkh1p were recently found to be involved in the uptake step of endocytosis (deHart *et al*., 2002; Friant *et al*., 2001). A double *pkh1-ts pkh2Δ* mutant exhibits an α-factor uptake defect similar to that observed in the *myo5-S357A myo3Δ* mutant (deHart *et al*., 2002; Friant *et al*., 2001; figure 5.2), making them interesting candidates to be type I myosin upstream factors.

***5.1.1.4.2 Pkh1p and Pkh2p specifically interact with Myo5p* in vivo**

To further investigate the possible physiological relevance of the finding that Pkh2p is able to phosphorylate the GST-Myo5-TEDSp protein *in vitro*, the ability of Pkh2p and its homologue Pkh1p to interact with the Myo5p head *in vivo* was tested using a yeast two-hybrid assay. Consistent with the *in vitro* phosphorylation data, the LexA DNA binding domain fused to the complete motor head of Myo5p (LexA-Myo5-Hp) was able to specifically induce β-galactosidase transcription when co-expressed with the B42 transcriptional activator fused to either Pkh1p or Pkh2p (figure 5.14A). This interaction seemed to be highly specific because no significant transcriptional activation could be detected when the LexA-Myo5-Hp was co-expressed with B42 fusions bearing any of six other kinases that were previously reported to play a role in the endocytic uptake (Ypk1p, Ark1p, Prk1p, Yck1p or Pkc1p) (figure 5.14A; deHart *et al*., 2002; Friant *et al*., 2000; Watson *et al*., 2001). Ypk2p (Ykr2p) was additionally tested, since it is a homologue to Ypk1p (Casamayor *et al.*, 1999). Surprisingly, no interaction of the Myo5p head with Cla4p, one of the yeast PAKs, was detected (figure 5.14A). Likewise, Ire1p, the kinase that gave the strongest signal in the chip assay, did not interact with Myo5p (compare figure 5.13C to 5.14A). Ire1p is an endoplasmic reticulum resident protein and therefore it is not likely to be involved in myosin-I dependent processes (Cox *et al*., 1993).

As described in the beginning of this chapter, deletion of *MYO5* causes synthetic growth defects when combined with mutations in genes encoding proteins that closely cooperate with the myosins-I *in vivo* (Geli *et al*., 2000; Soulard *et al*., 2002). As shown in figure 5.14B, deletion of *MYO5* in a temperature-sensitive *pkh1 pkh2* mutant (*pkh1-ts pkh2Δ*, Friant *et al*., 2001) strongly exacerbated the growth defect of this strain, demonstrating a genetic interaction of these genes.

The two-hybrid and the genetic data thus validated the results obtained in the biochemical screen suggesting that a signalling pathway alternative to the Cdc42p-PAKs is involved in the activation of Myo5p for its endocytic function, which might involve the kinases Pkh1p and Pkh2p.

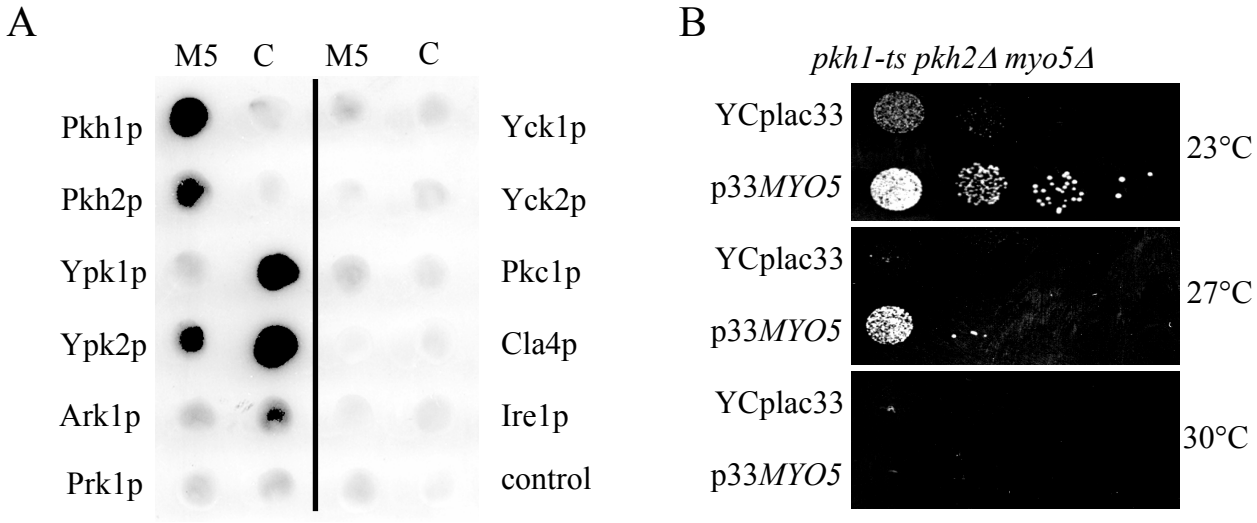

Figure 5.14: Myo5p and the yeast kinases Pkh1p and Pkh2p interact *in vivo*

(A) To test two-hybrid interactions, EGY48 cells bearing the β-galactosidase reporter on plasmid pSH18-34 and expressing the Myo5p head fused to the LexA binding domain (pEG202*myo5*-H; M5) or a control (pRFM-1; C) together with the genes encoding the indicated kinases fused to the B42 transcriptional activator (pJG4-5*PKH1*, Pkh1p; pJG4-5*PKH2*, Pkh2p; pJG4-5*YPK1*, Ypk1p; pJG4-5*YPK2*, Ypk2p; pJG4-5*ARK1*, Ark1p; pJG4-5*PRK1*, Prk2p; pJG4-5*YCK1*, Yck1p; pJG4-5*PKC1*, Pkc1p; pJG4-5*CLA4*, Cla4p; pJG4-5*IRE1*, Ire1p) or the B42 alone (pJG4-5; control) were spotted on X-Gal containing plates. Yeast cells were grown at 30°C for 2 days. An interaction was scored as positive when cells developed a blue (black) colour clearly before the corresponding controls. (B) The *pkh1-ts pkh2Δ myo5Δ* mutant (SCMIG605; *pkh1-ts pkh2Δ myo5Δ*) was either transformed with the empty plasmid (YCplac33) or the same plasmid carrying the *MYO5* gene (p33*MYO5*). Saturated cultures of the strains were diluted to 2.5 x 10^7 cells/ml and 1 to 10 dilution series were made for each strain. 5µl of each dilution was spotted on minimal media and the cells were grown for 2 days at the indicated temperatures.

Pkh1p and Pkh2p are activated by sphingoid bases *in vitro* (Friant et al., 2001). Moreover, overexpression of Pkh1p and Pkh2p suppresses the uptake defect of a *lcb1-100* mutant (Friant *et al*., 2001). *LCB1* encodes a serine palmitoyltransferase, which catalyzes the first step in sphingoid base synthesis (Zanolari *et al.*, 2000). These data indicated that a signal transduction pathway, which includes the sphingoid base-activated Pkh1p and Pkh2p kinases, regulates endocytic internalization in yeast. If Myo5p would be the only or more relevant target of the Pkh1p and Pkh2p kinases, expression of Myo5-S357Ep should be able to rescue the endocytic defects of the *pkh1-ts pkh2Δ* and the *lcb1-100* mutants. As shown in figure 5.15 though, Myo5-S357Ep was not able to suppress their endocytic defects, possibly indicating that the yeast type I myosins are not the only Pkh1p and Pkh2p targets.

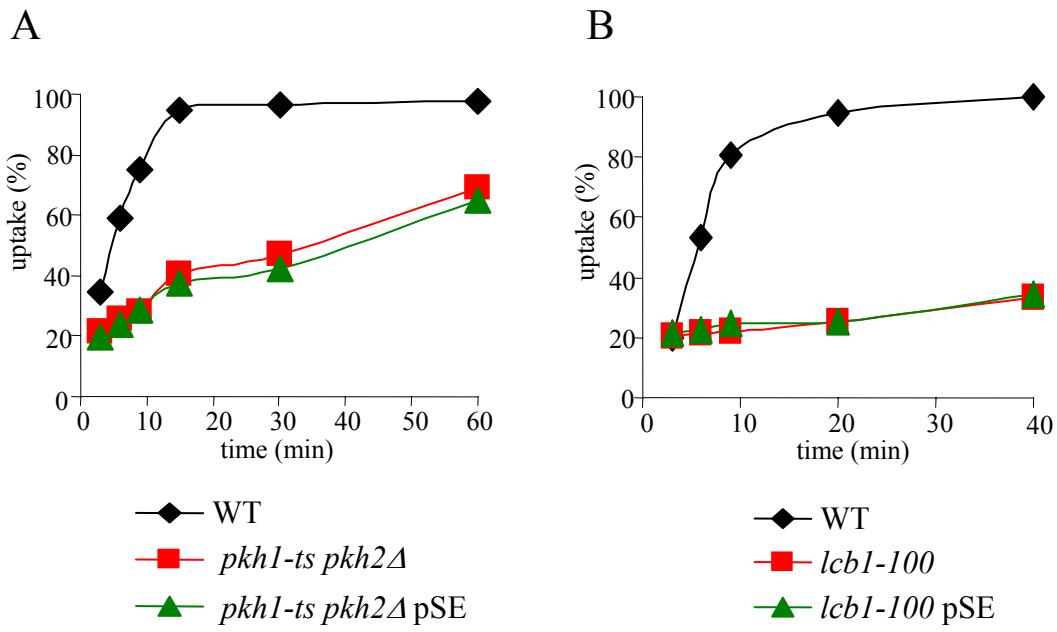

Figure 5.15: Myo5-S357Ep does not suppress the requirement for sphingoid base and the kinases Pkh1p/Pkh2p in endocytosis

(A) Uptake kinetics of ^{35}S-labelled α-factor pheromone of a wild-type (SCMIG603) and the *pkh1-ts pkh2Δ* strain (SCMIG604) either carrying an empty plasmid (YCplac33; *pkh1-ts pkh2Δ*) or the same plasmid carrying the *myo5-S357E* gene (p33*myo5-S357E*; *pkh1-ts pkh2Δ* pSE). Cells were preincubated at 30°C for 15 minutes, ^{35}S-labelled α-factor pheromone was added and the internalization was allowed for the indicated times at 30°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points. (B) Uptake kinetics of ^{35}S-labelled α-factor pheromone of a wild-type (SCMIG19) and the *lcb1-100* strain (SCMIG69) carrying either an empty plasmid (YCplac33; *lcb1-100*) or the same plasmid bearing the *myo5-S357E* gene (p33*myo5-S357E*; *lcb1-100* pSE). Cells were preincubated at 37°C for 15 minutes, ^{35}S-labelled α-factor pheromone was added and the internalization was allowed for the indicated times at 37°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points.

5.2 Analysis of the regulation of myosin-I-induced actin polymerization by phosphorylation

5.2.1 Yeast casein kinase II (CKII) phosphorylates serine 1205 in the Myo5p tail domain

5.2.1.1 Myosin-I-induced actin polymerization is regulated by phosphorylation

Recent data indicated that the fungal and protozoal type I myosins are able to induce Arp2/3-dependent actin polymerization (Evangelista *et al*., 2000; Geli *et al*., 2000; Jung *et al*., 2001; Lechler *et al*., 2000; Lee *et al*., 2000; for more detail see Introduction). Our laboratory developed an *in vitro* assay, which demonstrates that a C-terminal fragment of Myo5p including the TH2, SH3 and acidic domains (Myo5-Cp) is able to induce Arp2/3-dependent actin polymerization in the presence of ATP and yeast cytosol. Briefly, glutathione Sepharose beads coated with a GST-Myo5-Cp recombinant fusion protein are incubated in the presence of a yeast extract, ATP and rhodamine-labelled actin. Actin polymerization can be visualized by fluorescence microscopy (Geli *et al*., 2000; Idrissi *et al*., 2002; for more detail see Introduction).

Interestingly, the optical analysis of this process revealed that, although the beads are homogeneously covered by GST-Myo5-Cp, the rhodamine-actin signals appeared as discrete patch-like structures (figure 5.16). Further, even though neither the Arp2/3 complex nor actin are limiting in the assay, dilution of the yeast extract resulted in decrease of the number of actin patches per bead surface (Idrissi *et al.*, 2002). These results indicate that initiation of the process might be regulated by cytosolic components.

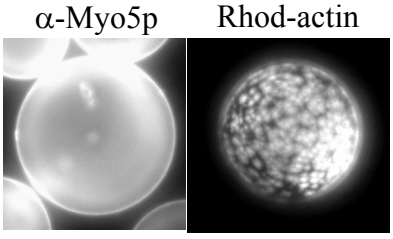

Figure 5.16: Myo5-Cp induces actin polymerization in discrete patches

Fluorescence micrographs of GST-Myo5-Cp-coated glutathione Sepharose beads either decorated with an α-Myo5p antibody and a FITC-conjugated secondary antibody (α-Myo5) or incubated with an extract from a wild-type yeast strain (SCMIG19) and 1μM rhodamine-labelled actin (Rhod-actin). These data are taken from Idrissi *et al.* (2002).

Interestingly, pharmacological analysis of the process revealed that the presence of phosphatase inhibitors in the polymerization assay significantly inhibited the process whereas the presence of kinase inhibitors slightly activated it (figure 5.17A). These results suggested that a kinase may negatively control one or more of the proteins involved in the process and dephosphorylation of this or these component/s might be required to initiate the assembly of the actin patches. The finding that Myo5p is not only phosphorylated at the TEDS site in the head domain (figure 5.12) opened the question whether Myo5p itself gets phosphorylated in the tail domain in order to regulate its function in actin polymerization (figure 5.17B).

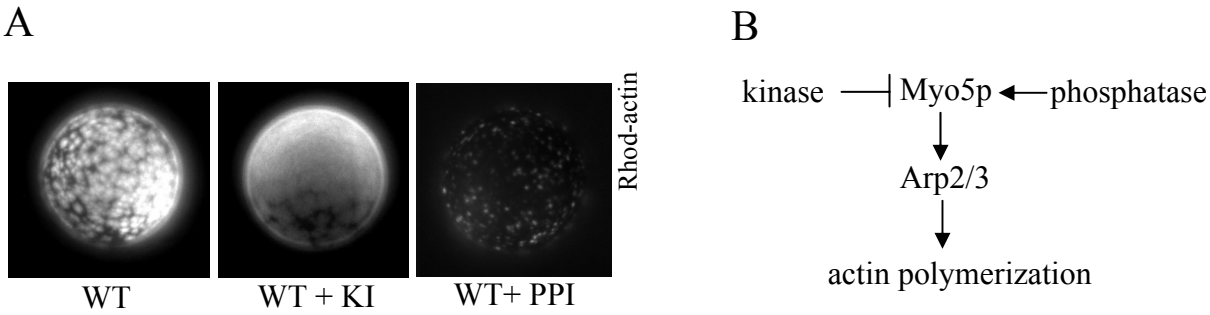

Figure 5.17: Myosin-I induced actin polymerization seems to be negatively regulated by phosphorylation

(A) Fluorescence micrographs of actin *foci* formed on beads coated with the GST-Myo5-Cp protein. The beads were incubated with an extract from a wild-type yeast strain (SCMIG19) and 1μM rhodamine-labelled actin either in the absence (WT) or in the presence of kinase inhibitors (WT+KI) or phosphatase inhibitors (WT+ PPI). (B) Schematic drawing of the working hypothesis that Myo5p itself might be phosphorylated at its tail domain, thereby being unable to induce Arp2/3-dependent actin polymerization.

5.2.1.2 Myo5p is phosphorylated at serine 1205

5.2.1.2.1 Serine 1205 of Myo5p is phosphorylated in vitro

To investigate whether the C-terminal domain of Myo5p is phosphorylated in the *in vitro* actin polymerization assay, the GST-Myo5-Cp-coated glutathione Sepharose beads were incubated with yeast extract and radiolabelled ATP. As shown in figure 5.18, the GST-Myo5-Cp protein is indeed phosphorylated *in vitro* (figure 5.18B, lower panel, "4"). Myo5p tail truncations mapped the phosphorylation site to the very C-terminus, since a small fragment carrying only the C-terminal part of the TH2 domain and the acidic domain (aa 1142-1219) was still phosphorylated in this assay (figure 5.18B, lower panel, "2").

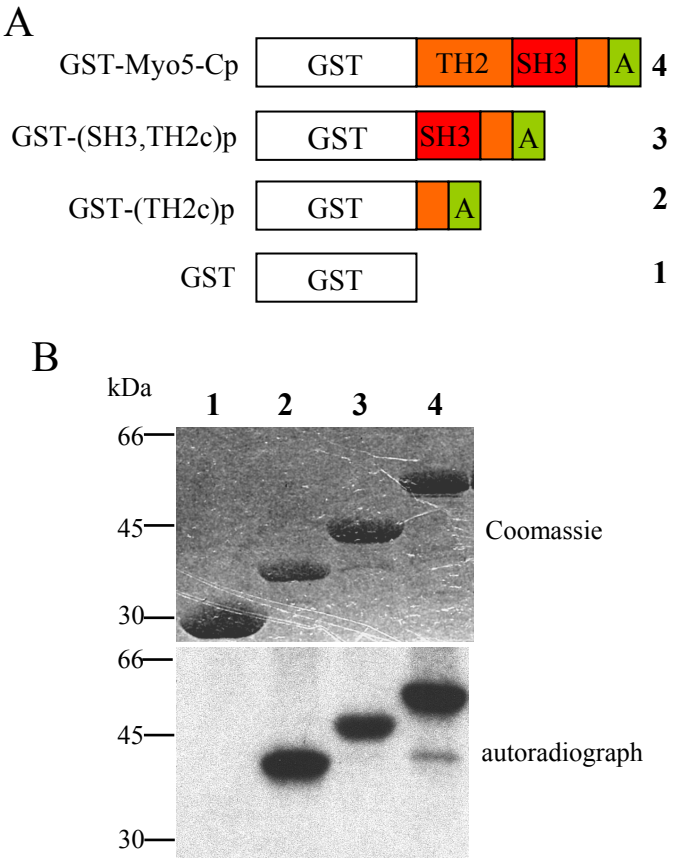

Figure 5.18: The Myo5p tail domain is phosphorylated *in vitro*

(A) Schematic drawings of the GST-fusion proteins examined in (B). (B) Either GST alone (1), GST-(TH2c)p (2), GST-(SH3,TH2c)p (3) or GST-Myo5-Cp (4) were bound to glutathione Sepharose beads and incubated with extract from a wild-type yeast strain (SCMIG19) and γ-^{32}P-ATP for 30 minutes at 26°C. Proteins were subjected to SDS-PAGE electrophoresis (Coomassie) and analysed by autoradiography.

To identify the exact phosphorylation site, the C-terminal sequence of Myo5p was scanned for phosphorylation motifs for known kinases. This search identified serine 1205 as a possible target of the yeast casein kinase II (figure 5.19).

The consensus motif for the yeast casein kinase II (CKII) consists of a serine or threonine surrounded by acidic amino acids that may extent from positions –2 to +5 (Meggio *et al*., 1994). An acidic residue at +3 was shown to be most important but not crucial in an otherwise acidic context (Meggio *et al*., 1994). In the case of Myo5p, serine 1205 is located in an acidic stretch that extends from amino acid –1 to +4, giving an excellent putative CKII phosphorylation site (figure 5.19A). If this serine were the site of phosphorylation in the bead assay, exchanging the residue to a non-phosphorylatable amino acid should abolish phosphorylation. Indeed, exchanging serine 1205 into alanine or glutamate abolished the phosphorylation of GST-Myo5-Cp (figure 5.19B). This result thus suggests that serine 1205 of Myo5p is phosphorylated *in vitro*.

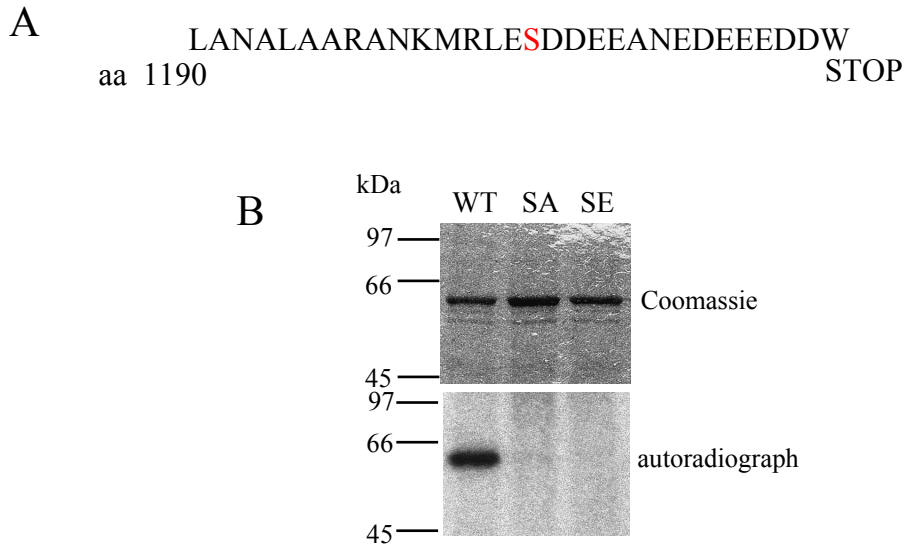

Figure 5.19: Serine 1205 of Myo5p is phosphorylated *in vitro*

(A) The C-terminal sequence of Myo5p from amino acids 1190 to the STOP codon. Serine 1205 is shown in red. (B) GST-Myo5-Cp (WT), GST-myo5-S1205A-Cp (SA) or GST-myo5-S1205E-Cp (SE) were bound to glutathione Sepharose beads and incubated with an extract from a wild-type yeast strain (SCMIG19) and γ-^{32}P-ATP for 30 minutes at 26°C. Proteins were subjected to SDS-PAGE electrophoresis (Coomassie) and analysed by autoradiography.

5.2.1.2.2 In vivo *evidence for serine 1205 phosphorylation*

In order to determine whether serine 1205 of Myo5p is phosphorylated *in vivo*, cells expressing wild-type Myo5p or Myo5-S1205Ap were incubated in the presence of radiolabelled PO_4^{3-}. Cells were subsequently lysed, Myo5p or Myo5-S1205Ap was immunoprecipitated and the incorporation of radioactivity was determined by autoradiography. Interestingly, even though the radioactive signal associated to equivalent fractions of wild-type and mutant precipitates did not seem to drop in the Myo5-S1205Ap sample (figure 5.20), immunoblot analyses revealed that at least 10-fold more Myo5-S1205Ap had been immunoprecipitated compared to wild-type Myo5p sample (figure 5.20). This result indicated that the level of phosphorylation relative to the total immunoprecipitated myosin was indeed significantly reduced in the Myo5-S1205Ap mutant compared to the wild-type. Further, the experiment suggested that Myo5-S1205Ap is either more stable or better expressed than the wild-type Myo5p or the mutant protein is more efficiently immunoprecipitated (see Discussion).

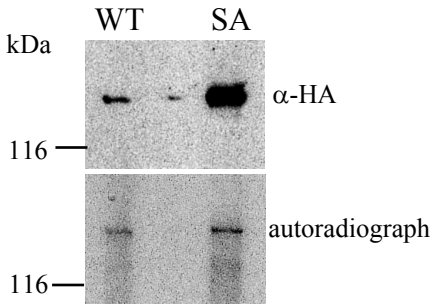

Figure 5.20: *In vivo* phosphorylation of wild-type Myo5p compared to Myo5-S1205Ap

Cells deleted for Myo5p carrying plasmid YCplac33 containing either the *MYO5HA$_3$* (p33*MYO5HA$_3$*; M5) or the *myo5-S1205AHA$_3$* (p33*myo5-S1205A HA$_3$*; SA) gene, were radiolabelled *in vivo* with $^{32}PO_4^{3-}$ for 30 minutes. Cells were subsequently lysed and immunoprecipitated with a Myo5p-tail-specific antibody bound to ProtA-Sepharose beads. Half of the Immunoprecipitates were separated by SDS-PAGE and analysed by immunoblot (upper panel) using a rat α-HA antibody (α–HA) or by autoradiography (lower panel).

5.2.1.3 Casein kinase II (CKII) interacts with Myo5p

5.2.1.3.1 CKII phosphorylates the Myo5p tail in vitro

If CKII phosphorylates the Myo5p tail domain at serine 1205, a yeast extract lacking CKII activity should no longer be able to phosphorylate the Myo5p tail. The yeast CKII is a heterotetramer composed of two catalytical subunits, encoded by *CKA1* and *CKA2*, and two regulatory subunits, encoded by *CKB1* and *CKB2* (Glover, 1998). Deletion of either one of the catalytic subunits, *CKA1* or *CKA2*, does not cause an obvious phenotype under standard growth conditions, but deletion of both genes is lethal (Padmanabha *et al*., 1990). In order to investigate whether the cytosolic kinase activity that phosphorylates the Myo5p serine 1205 (figure 5.19) is CKII, the GST-Myo5-Cp-containing beads were incubated with an extract from a temperature-sensitive CKII mutant (*cka1Δ cka2-ts*, kindly provided by Dr. C.V.C.Glover; Glover, 1998). As shown in figure 5.21A, the GST-Myo5-Cp tail fragment was no longer phosphorylated under these conditions. Moreover, incubation of the GST-Myo5-Cp with wild-type yeast extract in the presence of heparin, an inhibitor of CKII (Rigobello *et al*., 1982), significantly reduced phosphorylation of Myo5p (figure 5.21B).

In order to further proof that CKII phosphorylates serine 1205 of Myo5p, beads carrying the Myo5p tail fragment were incubated with purified CKII. As shown in figure 5.21C, purified kinase efficiently phosphorylated the tail of Myo5p, but mutation of serine 1205 into alanine completely abolished this phosphorylation.

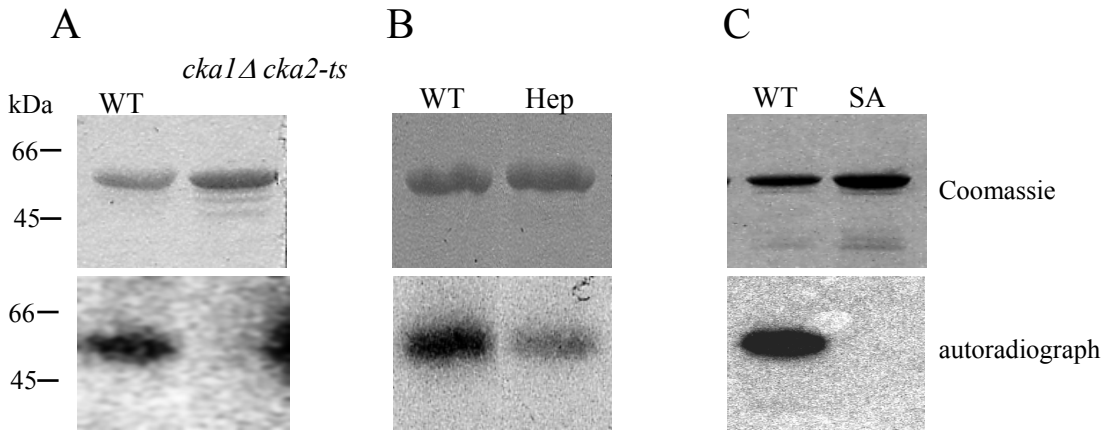

Figure 5.21: CKII phosphorylates serine 1205 of the Myo5p tail *in vitro*

(A) GST-Myo5-Cp was bound to glutathione Sepharose beads and incubated with an extract from either a wild-type (SCMIG19, WT) or from a *cka1Δ cka2Δ* p*cka2-ts* (SCMIG629; *cka1Δ cka2-ts*) strain and γ-^{32}P-ATP for 30 minutes at 30°C. Proteins were subjected to SDS-PAGE electrophoresis (Coomassie) and analysed by autoradiography. (The reported wild-type is not the isogenic wild-type for the *cka* mutant strain) (B) GST-Myo5-Cp was bound to glutathione Sepharose beads and incubated with an extract from a wild-type yeast strain (SCMIG19) either alone (WT) or in the presence of 0.25 μg/ml heparin (Hep) and γ-^{32}P-ATP for 30 minutes at 30°C. Proteins were subjected to SDS-PAGE (Coomassie) and analysed by autoradiography. (C) GST-Myo5-Cp (WT) or GST-Myo5-S1205A-Cp (SA) were bound to glutathione Sepharose beads and incubated with 50 U of recombinant human CKII and γ-^{32}P-ATP for 30 minutes at 30°C. Proteins were subjected to SDS-PAGE (Coomassie) and analysed by autoradiography.

5.2.1.3.2 CKII binds to Myo5p in a two-hybrid approach

The previous data strongly indicated that CKII is the kinase that phosphorylates serine 1205 in the Myo5p tail domain *in vitro*. To further investigate whether CKII and Myo5p interact *in vivo*, the two-hybrid assay was used. Consistent with our previous data, the LexA DNA binding domain fused to the whole tail of Myo5p was able to induce the transcription of a β-galactosidase reporter gene when it was co-expressed with the B42 transcriptional activator fused to the regulatory subunits of CKII, *CKB1* and *CKB2* (figure 5.22).

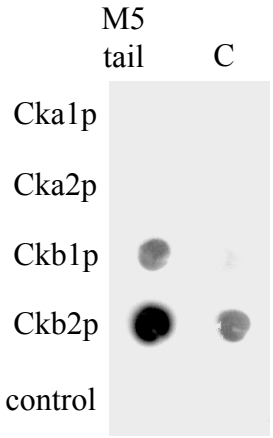

Figure 5.22: CKII and Myo5p interact in a two-hybrid assay *in vivo*

EGY48 cells bearing the inducible β-galactosidase reporter on plasmid pSH18-34 and expressing the Myo5p tail fused to the LexA binding domain (pEG202*myo5*-T; M5 tail) or a control (pRFM-1; C) together with the genes encoding the indicated kinases fused to the B42 transcriptional activator (pJG4-5*CKA1*, Cka1p; pJG4-5*CKA2*, Cka2p; pJG4-5*CKB1*, Ckb1p; pJG4-5*CKB2*, Ckb2p) or the B42 alone (pJG4-5; control) were spotted on X-Gal containing plates. Yeast cells were grown at 30°C for 2 days. An interaction was scored as positive when cells developed a blue (black) colour clearly before the corresponding control.

5.2.2 Phosphorylation of Myo5p serine 1205 by casein kinase II might negatively regulate actin polymerization and endocytosis

5.2.2.1 A negative charge at position 1205 of Myo5p decreases the number of myosin-I induced actin patches *in vitro*

Since myosin-I induced actin polymerization appears to be negatively regulated by phosphorylation (figure 5.17) and the Myo5p tail is phosphorylated at serine 1205 *in vitro* by CKII (figure 5.21), the question arose whether this phosphorylation event might negatively regulate myosin-I-induced actin polymerization. To investigate this matter, serine 1205 of Myo5p was mutated into alanine or glutamate in the GST-Myo5-Cp protein. Wild-type and mutant proteins were bound to glutathione Sepharose beads and analysed for their ability to induce actin polymerization. As shown in figure 5.23, mutation of serine into glutamate led to a decrease of actin *foci* on the bead surface (figure 5.23A and B).

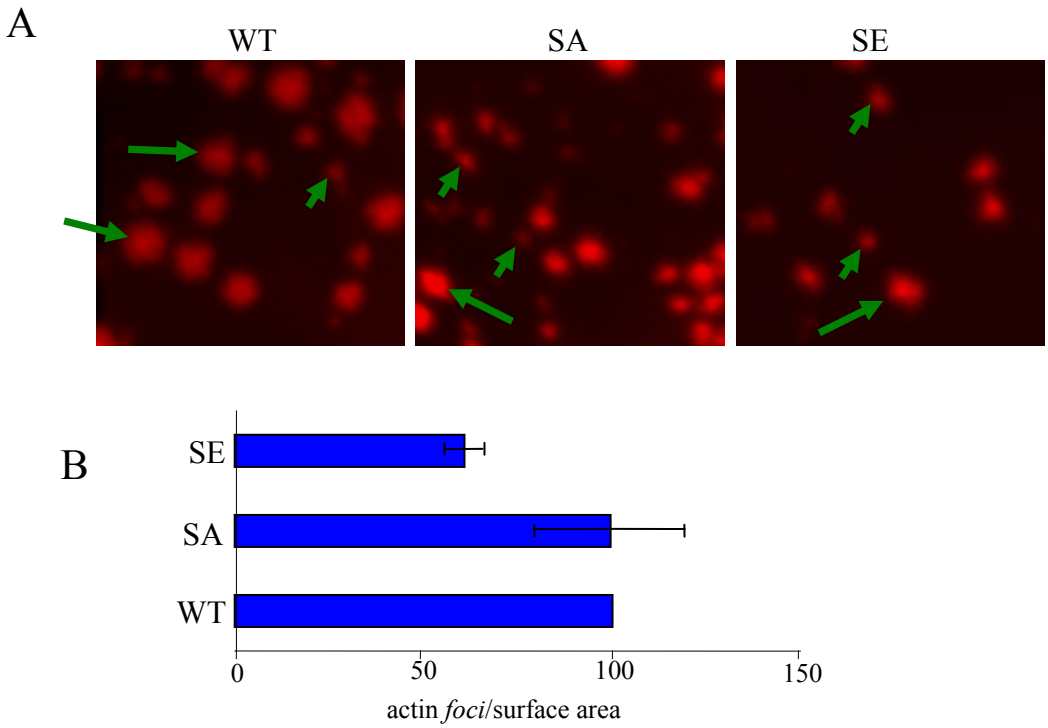

Figure 5.23: Mutation of serine 1205 of Myo5p into glutamate seems to inhibit myosin-I-induced actin assembly

(A) GST-Myo5-Cp (WT), GST-Myo5-S1205A-Cp (SA) or GST-Myo5-S1205E-Cp (SE) proteins were bound to glutathione Sepharose beads and incubated with an extract from a wild-type yeast strain (SCMIG19) and 1µM rhodamine-labelled actin for 15 minutes at 26°C. Arrows indicate bigger actin *foci*, smaller *foci* are indicated by arrow heads. (B) GST-Myo5-Cp (WT), GST-Myo5-S1205A-Cp (SA) or GST-Myo5-S1205E-Cp (SE) proteins were bound to glutathione Sepharose beads and incubated with an extract from a wild-type yeast strain (SCMIG19) and 1µM rhodamine-labelled actin. The assays were performed for 15 minutes at 24°C or 26°C. Two areas of 6 x 6 µm were defined per bead and actin *foci* within these areas were counted. For each experiment at least 6 beads were counted. The number of the wild-type was set to 100%, the numbers of the mutants were calculated accordingly. The graphs show the average of 6 independent experiments.

In contrast, mutation of the same residue into alanine did not significantly influence the number of these actin *foci* per bead surface in the assay (figure 5.23B). However, both mutations (*MYO5-S1205A* and *MYO5-S1205E*) increased the frequency of small actin *foci*, suggesting that both, phosphorylation and dephosphorylation, might be required for growth of the actin-containing structures (figure 5.23A).

5.2.2.2 Depletion of *CKA2* accelerates the endocytic uptake

As reported in the last chapters, the Myo5p serine 1205 appears to be phosphorylated by casein kinase II (CKII). Consistent with a possible role of this event as a negative regulator of Myo5p-induced actin polymerization, it was shown that mutation of serine 1205 into glutamate led to a significant decrease in myosin-I-induced actin polymerization *in vitro*. Work from our laboratory indicated that the ability of the yeast type I myosins to induce actin polymerization is required for their role in endocytosis (Geli *et al*., 2000; Idrissi *et al*., 2002). Therefore, it seemed likely that phosphorylation of the Myo5p tail by CKII might negatively regulate endocytosis.

Consistent with this hypothesis, deletion of one of the catalytic subunits of CKII, *CKA2*, led to a significant increase in the initial α-factor uptake rate and caused rather sigmoid kinetics (figure 5.24A). This effect seemed to be specific for *CKA2* since deletion of *CKA1* did not seem to significantly alter the uptake kinetics (figure 5.24A).

If CKII would negatively regulate endocytosis, mutation of this kinase might not only lead to an increase in ligand-induced but also constitutive internalization of the receptor. As a consequence, the steady state distribution of the α-factor receptor might change with less receptor being available at the plasma membrane (figure 5.24B, lower panel). Consistent with this hypothesis, significantly less α-factor bound to *cka2Δ* cells compared to *cka1Δ* cells (figure 5.24C).

Taken together, the reported data support a role of at least one of the catalytic subunits of CKII, Cka2p, as a negative regulator of endocytosis in yeast.

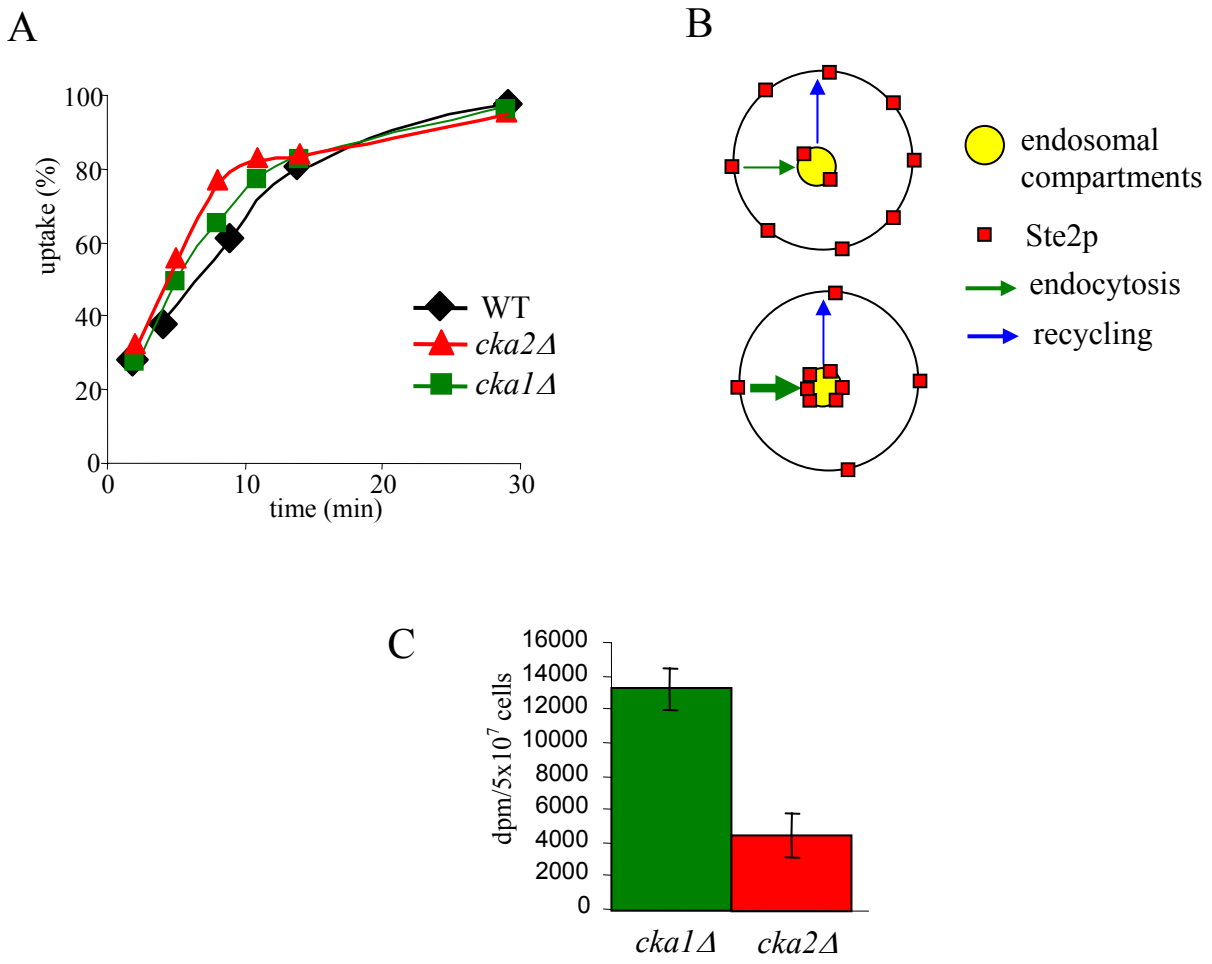

Figure 5.24: Deletion of one of the catytical subunits of CKII, *CKA2*, increases the α-factor internalization rate

(A) Uptake kinetics of ^{35}S-labelled α-factor pheromone of wild-type (SCMIG50; WT), *cka1Δ cka2Δ* p*CKA1* (SCMIG553; *cka2Δ*) and *cka1Δ cka2Δ* p*CKA2* (SCMIG549; *cka1Δ*) strains. Cells were preincubated at 37°C for 15 minutes, ^{35}S-labelled α-factor pheromone was added and the internalization was allowed for the indicated times at 37°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points. (The reported wild-type is not the isogenic wild-type for the *cka* mutant strains) (B) Schematic drawing of the wild-type Ste2p localization (upper panel) and the effect of increased endocytosis (green arrow) on the steady state distribution (lower panel) of the α-factor receptor Ste2p (red square). (C) Radioactive counts (dpm) for pH6 washes, which indicate the total cell-bound α-factor, for time point 3 min are given for the *cka1Δ cka2Δ* p*CKA1* (SCMIG553; *cka2Δ*) and *cka1Δ cka2Δ* p*CKA2* (SCMIG549; *cka1Δ*) strains.

5.2.2.3 Endocytosis and actin cytoskeleton polarization are not affected by mutation of Myo5p serine 1205

In order to investigate whether phosphorylation of serine 1205 in the full-length Myo5p might mediate the inhibitory effect of CKII in the endocytic uptake, this amino acid was mutated into alanine (S1205A) or glutamate (S1205E) in order to mimic the unphosphorylated or the phosphorylated states, respectively. Myosin-I double deletion strains bearing the wild-type or mutant *MYO5* genes on centromeric plasmids (for details see Materials and Methods) exhibited a wild-type-like growth-rate and were able to grow at all temperatures (data not shown) and all

mutants were expressed similar to wild-type levels (figure 5.25A). Neither the *myo5-S1205A myo3Δ* nor the *myo5-S1205E myo3Δ* mutant displayed a defect in the uptake step of endocytosis (figure 5.25B). Likewise, the second type I myosin function, polarization of the actin cytoskeleton, was unaffected (figure 5.25C), possibly indicating that type I myosins are not the only CKII targets for their function in actin assembly and/or endocytosis.

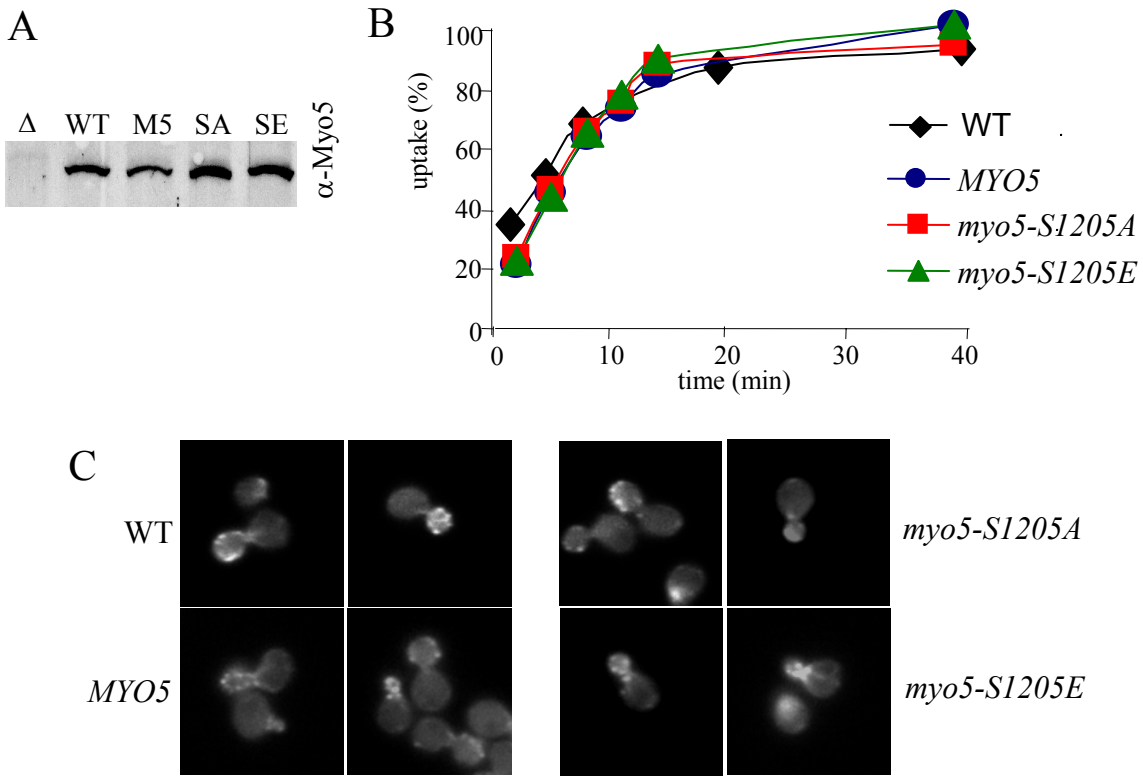

Figure 5.25: Myo5p tail phosphorylation itself does not seem to affect endocytosis and actin cytoskeleton polarization

(A) Equal amounts of protein extracts from *myo5Δ* (SCMIG51; Δ), wild-type (SCMIG19; WT), p*MYO5 myo3Δ* (SCMIG590; *MYO5*), p*myo5-S1205A myo3Δ* (SCMIG591; SA) and p*myo5-S1205E myo3Δ* (SCMIG592; SE) strains were loaded and proteins were probed with a Myo5p-tail-specific antibody (α-Myo5p). (B) Uptake kinetics of ^{35}S-labelled α-factor pheromone of wild-type (SCMIG19; WT), p*MYO5 myo3Δ* (SCMIG590; *MYO5*), p*myo5-S1205A myo3Δ* (SCMIG591; *myo5-S1205A*) and p*myo5-S1205E myo3Δ* (SCMIG592; *myo5-S1205E*) strains. Cells were pulsed with ^{35}S-labelled α-factor pheromone for 45 minutes at 0°C and chased for the indicated times at 23°C. The graphs show the percentage of cell-associated counts that have been internalized at the indicated time points. (C) Fluorescence micrographs showing wild-type (SCMIG19; WT), p*MYO5 myo3Δ* (SCMIG590; *MYO5*), p*myo5-S1205A myo3Δ* (SCMIG591; *myo5-S357A*) and p*myo5-S1205E myo3Δ* (SCMIG592; *myo5-S357E*) cells stained with TRITC-phalloidin.

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6 Discussion

6.1 p21-activated kinases (PAKs) are not the only TEDS site kinases

The data presented in the first part of this study indicated that Myo5p TEDS site phosphorylation is required for two *in vivo* processes that require type I myosin activity, endocytosis and actin cytoskeleton polarization. However, this study shows that the kinases that had previously been found to phosphorylate type I myosins *in vitro*, the p21-activated kinases (PAKs), are only required for actin cytoskeleton polarization, but not for the uptake step of endocytosis. The PAK upstream factor Cdc42p is also dispensable for internalization, emphasizing that PAKs do not play a role in this process. These data thus indicate that uptake at the plasma membrane might require activation of myosins-I by other kinases. Consistently, an *in vitro* screen revealed that 16 additional kinases can phosphorylate a fragment of the Myo5p head that contains the TEDS site. One of these kinases, Pkh2p, and its homologue Pkh1p are required for endocytosis and both physically and genetically interact with Myo5p.

6.1.1 TEDS site phosphorylation and dephosphorylation are probably required for myosin-I function in endocytosis and in the actin cytoskeleton polarization

The finding that mutation of the TEDS site serine into alanine in the yeast type I myosin Myo3p does not rescue lethality of the *myo3Δ myo5Δ* double mutant, while mutation into aspartate does, already indicated that TEDS site phosphorylation might play a role in type I myosin function in budding yeast (Wu *et al*., 1997). However, Wu and co-workers could not exclude the possibility that *myo3-S357A* mis-functioning was due to a low or absent expression of the mutant protein or due to its inability to reach the plasma membrane. Here, data were presented that support the hypothesis that phosphorylation of the type I myosin Myo5p TEDS site is required for its functions *in vivo*.

A non-phosphorylatable myosin-I double mutant strain, *myo5-S357A myo3Δ*, was unable to internalize α-factor and did not polarize the actin cytoskeleton either (figures 5.2 and 5.3), two phenotypes also installed in yeast cells depleted of type I myosin activity (Geli and Riezman, 1996; Goodson *et al*., 1996). As indicated by the amount of Myo5-S357Ap present in the cell, these effects were most likely not caused by misfolding of the mutant protein and its subsequent degradation (figure 5.2). Consistent with the view that the serine to alanine mutation in the TEDS site of the myosins-I does not cause major unfolding of the protein, it

was found that the analogous mutant in the *Acanthamoeba castellanii* myosin IC strongly resembled the unphosphorylated myosin isoform at the biochemical level (Wang *et al*., 1998). The myosin mutant could be purified following the same protocol as for the wild-type protein and it was still able to hydrolyze ATP with an ATPase activity similar to the unphosphorylated myosin IC (0.98 s^{-1} compared to 1.37 s^{-1}). Likewise, the fact that Myo5-S357Ap could reach the plasma membrane supports the view that at least the domains required for membrane targeting were properly folded. Finally, we could also rule out the possibility that the slow growth and temperature-sensitive phenotype of the *myo5-S357A myo3Δ* strain might be caused by general sickness of the mutant cells since another myosin-I-independent trafficking event, transport of carboxypeptidase Y from the ER to the vacuole, was completely unaffected by this mutation (figure 5.6).

In order to mimic the phospho-serine state by charge, the Myo5p TEDS site serine was substituted by glutamate. Wang and co-workers had shown that a mutant *Acanthamoeba* myosin IC with glutamate at the corresponding position exhibited an ATPase activity (20.29 s^{-1}) and *in vitro* motility (0.098 μm/s) that strongly resembled the behaviours of the phosphorylated myosin (19.85 s^{-1} and 0.11 μm/s, respectively) (Wang *et al*., 1998). In contrast to the serine to alanine mutation, the *myo5-S357E myo3Δ* mutant exhibited nearly wild-type-like endocytic kinetics and was able to polarize the actin cytoskeleton (figures 5.2 and 5.3), implying that a negative charge at the TEDS site is sufficient to sustain at least some myosin functions.

All these data therefore strongly indicate that TEDS site phosphorylation, and thus activation of the ATPase, are required for the redundant functions of the two yeast type I myosins in endocytosis and actin cytoskeleton polarization (Geli and Riezman, 1996; Goodson *et al*., 1996).

The finding that Myo5p TEDS site phosphorylation is required for *in vivo* function is consistent with data from the *Dictyostelium discoideum* type I myosins. Novak and Titus have shown that substitution of the myoB TEDS site serine into alanine produced a protein that was unable to complement the defects of the myoA-/myoB- double mutant in pinocytosis and F-actin rearrangements (Novak and Titus, 1998), strongly resembling the data presented in this work.

In contrast to our findings and the results from *Dictyostelium*, TEDS site phosphorylation of the *Aspergillus nidulans* MyoA protein was not necessary to sustain the essential *in vivo* functions (Liu *et al*., 2001; Yamashita and May, 1998), as *Aspergillus* expressing MYOA bearing a serine to alanine mutation at the TEDS site was able to complement growth to wild-

type levels. Thus, phosphorylation of the TEDS site might not be required for all myosin-I functions. Interestingly, even though the *myo5-S357A myo3Δ* displayed a slow growth and temperature-sensitive phenotype, cells were still able to grow suggesting that the essential myosin-I function could be at least partially complemented by these mutant alleles (Wu *et al*., 1997).

On the other hand, it should be noted that the mutant allele that mimics phosphorylation, *myo5-S357E myo3Δ*, allowed only incomplete α-factor internalization and actin cytoskeleton polarization (figures 5.2 and 5.3). Similarly, Liu and co-workers have shown that hyphal branching and growth in liquid culture of *Aspergillus* cells were not only delayed in a non-phosphorylatable TEDS site mutant strain (MYOA-S371A), but also in a MYOA-S371E mutant strain (Liu *et al*., 2001). It is possible that the Myo5-S357Ep mutant did not completely imitate the phosphorylated wild-type Myo5p isoform and that, therefore, uptake kinetics and actin polarization are not completely restored. However, this possibility seems unlikely because, as previously mentioned, the *Acanthamoeba* myosin IC TEDS site serine to glutamate mutant strongly resembled the phosphorylated isoform (Wang *et al*., 1998). Thus, these data rather imply that dephosphorylation of the TEDS site serine is also required for proper myosin-I function in yeast. It is tempting to speculate that the observed lower plateau in the α-factor internalization kinetics in the *myo5-S357E myo3Δ* cells compared to *MYO5 myo3Δ* cells might be due to a defect in the recycling of the endocytic machinery, which recognizes Ste2p, to the plasma membrane (Wiederkehr *et al*., 2000). The finding that a TEDS site serine to glutamate mutation of the *Aspergillus* type I myosin MyoAp led to accumulation of intracellular membranes was interpreted as a constitutive activation of endocytosis (Yamashita and May, 1998). However, it is also possible that the accumulation of membranes was the result of a failure to recycle the endocytic machinery back to the plasma membrane. Consistent with this, type I myosins in *Dictyostelium* have been found to be involved in the recycling of endocytosed material back to the cell surface (Neuhaus and Soldati, 2000). If dephosphorylation of Myo5p is required for efficient recycling in yeast, the uptake machinery that recognizes the Ste2p cytosolic tail might be exhausted upon ligand induced internalization in the *myo5-S357E myo3Δ* cells, which would then explain the inability of the mutant cells to internalize to totality the cell surface bound α-factor.

Taken together, data from this study indicate that TEDS site phosphorylation of the yeast type I myosins is required for their function in endocytosis and actin cytoskeleton polarization. Moreover, the data also suggest that dephosphorylation might be necessary for proper myosin-I function.

6.1.2 TEDS site phosphorylation appears to occur subsequent to plasma membrane recruitment

The presented data show that the Myo5-S357Ap mutant protein still seems to be localized to the plasma membrane, implying that TEDS site phosphorylation is not required for plasma membrane recruitment (figures 5.4 and 5.5). This finding is consistent with data from Novak and Titus, who demonstrated that TEDS site mutation did not lead to a mislocalization of the *Dictyostelium discoideum* type I myosin myoB (Novak and Titus, 1998). The depolarized distribution of the Myo5-S357Ap patches at the plasma membrane (figure 5.4) might indicate that TEDS site phosphorylation is required for correct Myo5p localization to certain plasma membrane subdomains. However, since proper localization of the yeast type I myosins requires actin (Anderson *et al*., 1998), it is more likely that the observed Myo5-S357Ap distribution just follows the depolarized actin patches in this mutant (figure 5.3).

The *Acanthamoeba castellanii* phospho-myosin IC is approximately 20-fold enriched at the contracting vacuole, compared to the filling vacuole (Baines *et al*., 1995), suggesting that myosin IC is phosphorylated at the moment the vacuole contracts. In addition, the myosin IB of this organism appeared greatly enriched at highly dynamic regions at the plasma membrane such as filopodia or phagocytic cups. This observation that the phosphorylated isoforms are enriched at the sites where myosins-I exert their functions, although TEDS site phosphorylation might not be required for proper protein localization, suggests that phosphorylation of the myosin-I TEDS site occurs locally. Type I myosins might thus be activated specifically where the myosin-I ATPase activity is required and dephosphorylation might occur shortly after the respective function has been carried out. Consistent with the idea of a local and transient phosphorylation, the Myo5p head domain, which is not recruited to its site of function, i.e, the plasma membrane (H.Groetsch, personal communication), is not phosphorylated *in vivo* (figure 5.12). Also consistent with this view, no obvious drop in the *in vivo* phosphorylation signal was observed when the Myo5-S357Ap mutant protein was compared to the wild-type protein in *in vivo* phosphorylation experiments (figure 5.12).

Further support for the idea that phosphorylation happens at the sites of myosins-I function comes from the localization of the putative TEDS site kinases at the plasma membrane. PAKs, which can phosphorylate the myosin-I TEDS site *in vitro* and might activate the myosins-I for their function in actin polarization *in vivo*, are localized to the plasma membrane and are activated by binding to membrane-bound and polarized Cdc42p-GTP (Peter *et al*., 1996; Holly and Blumer, 1999). Likewise, Pkh1p and Pkh2p, the putative activators of the myosins-I for

their endocytic function, have been localized to patch-like structures at the plasma membrane that might be the sites of endocytosis (Roelants *et al*., 2002).

Finally, evidence supporting the view that TEDS site phosphorylation and activation of the myosin ATPase might happen locally comes from the type VI myosins, the only other myosin class that is known to carry a phosphorylatable residue at the TEDS site, (for review see Buss *et al*., 2001b). Buss and co-workers have shown that the phosphorylation of the mammalian isoform increases with ongoing recruitment into membrane ruffles, which possibly indicates a recruitment-dependent phosphorylation event (Buss *et al*., 1998a). This phosphorylation is likely to be PAK-mediated phosphorylation of the TEDS site, since immunoprecipitated myosin VI was phosphorylated at the corresponding serine by a GST-PAK3 protein *in vitro* (Buss *et al*., 1998a).

6.1.3 PAKs and Cdc42p are dispensable for receptor-mediated endocytosis in yeast

6.1.3.1 PAKs and Cdc42p are required for actin cytoskeleton polarization

Previous data indicated the presence of a signal transduction pathway leading from the yeast Cdc42p to the PAKs, which would then phosphorylate the TEDS site of type I myosins, thus activating the ATPase of these molecular motors for their function in actin polarization (see Introduction for more detail). However, some results were inconsistent with the proposed hypothesis. In particular, *ste20 cla4* double mutant strains exhibited polarization defects only at high temperatures but not at physiologic temperature, implicating the yeast PAKs Ste20p and Cla4p only under heat shock conditions in actin cytoskeleton polarization (Holly and Blumer, 1999; Eby *et al*., 1998; Crvckova *et al*., 1995; Weiss *et al*., 2000) (see Introduction).

This study now shows that PAK mutants indeed have a striking defect in the actin cytoskeleton polarization even under standard growth conditions. After treatment with 100 µM of the Cla4as3p inhibitor 1NM-PP1, the *ste20Δ skm1Δ cla4-as3* mutant showed a depolarized actin cytoskeleton with enlarged and round cells at 23°C (figure 5.10). This phenotype is very similar to that observed in cells depleted of myosin-I or Cdc42p activity. A small percentage (approximately 1%) of the cells displayed a long-budded morphology, but the actin cytoskeleton of these cells had also been depolarized after treatment with the chemical (data not shown). In contrast to this observation, Weiss and co-workers had observed that an inactivated *ste20Δ cla4-as3* mutant exhibited a depolarized actin cytoskeleton only after heat shock (Weiss *et al*., 2000). The observed differences might be due to a tighter inactivation of the PAK activity by applying the four-fold amount of 1NM-PP1 as compared to Weiss and co-

workers. These authors already offered the possibility that the amount of inhibitor that they had applied might have left some residual PAK activity that was enough to establish actin polarization at permissive temperature but not enough to establish polarity under stress conditions. Another possibility is that Skm1p, which is not present in the studies done within this work, might have been sufficient to sustain polarization of the actin cytoskeleton at 25°C in the *ste20Δ cla4as3* strain used by Weiss and co-workers (Weiss *et al*., 2000).

Although this result supports the existence of a possible signal transduction pathway from Cdc42p to the PAKs and the type I myosins, this work could not definitely prove this hypothesis since Myo5-S357Ep did not suppress the requirement of PAKs for actin cytoskeleton polarization (figure 5.10). This might indicate that type I myosins are not the only PAK targets in yeast and suppression might require generation of PAK mutants that specifically affect myosin-I function.

6.1.3.2 The yeast PAKs and Cdc42p are not required for endocytosis

Evidence from mammalian cells indicates that Cdc42 and PAKs are involved in endocytosis. Cdc42 is required for endocytosis at the basolaterial surface of mammalian epithelial cells (Kroschewski *et al*., 1999), for phagocytosis (Lee *et al*., 2000) and for clathrin-dependent and -independent internalization pathways in different cell lines (Sabharanjak *et al*., 2002; McGavin *et al*., 2001; Sakr *et al*., 2001).

The evidence for a putative involvement of PAKs in endocytosis comes from the mammalian PAK1. It partially localizes to pinocytic structures and might be involved in the process of Rac-mediated and growth factor stimulated macropinocytosis (Dharmawardhane *et al*., 1999; Dharmawardhane *et al*., 1997; Dharmawardhane *et al*., 2000). Interestingly, Dharmawardhane and co-workers have also shown that expression of a kinase-inactive mutant (K299R) did not lead to a significant defect in macropinocytosis. Therefore the exact role of PAKs in this process remains to be clarified (Dharmawardhane *et al*., 2000).

Strikingly, the data from this thesis clearly indicate that in yeast, neither PAKs nor Cdc42p are required for the uptake step of endocytosis (figures 5.8-5.11). Although this seems to be in contrast to the findings in vertebrate cells, it is possible that Cdc42p and/or PAKs in yeast are required for later steps in endocytosis, e.g. transport steps between the endocytic compartments. Interestingly, one of the *cdc42* temperature-sensitive mutant strains, *cdc42-129*, exhibited a lower internalization kinetics plateau (figure 5.11), as has been observed for the *myo5-S357E myo3Δ* mutant (figure 5.2). As described above for the latter mutant, this lower plateau might indicate an involvement of Cdc42p in recycling.

Since type I myosin TEDS site phosphorylation was found to be required for functional uptake at the plasma membrane (figure 5.2), this result also implies that other kinases might activate the myosins for their function in endocytosis. This notion is consistent with the results that suggest that myosins-I are locally activated at the sites where they exert their function (see above). The signals that control actin polarization and endocytosis, and the cellular sites and the times at which these cellular processes might be turned on and off, are most likely different. Thus, different kinases and signalling cascades might be involved in the activation of the myosin-I for their different functions. Consistent with this hypothesis, it was found that 16 yeast kinases could phosphorylate a GST fusion protein carrying a fragment of the Myo5p head domain containing the TEDS site (figure 5.13). The fact that neither Cla4p nor Ste20p were found in this screen might be explained by the lack of GTP-Cdc42p or acidic phospholipids for activation of these kinases (Benton *et al*., 1997; Eby *et al*., 1998; Lamson *et al*., 2002; Peter *et al*., 1996).

6.1.3.3 Which TEDS site kinase activates the type I myosins for their function in endocytosis?

Based on the phenotypes described for the different kinase mutants, their described targets and/or intracellular localization, most of the kinases found to be capable of phosphorylating the Myo5p TEDS site in the *in vitro* screen are unlikely candidates to activate the type I myosins for their function in endocytosis (described in more detail in 5.1.1.4). The only exception to this is the sphingoid base-activated kinase Pkh2p (figure 5.13, red). First, a double mutant of Pkh2p and its homologue Pkh1p was shown to cause a defect in the uptake step of endocytosis (deHart *et al*., 2002; Friant *et al*., 2001), similar to the defect observed in the *myo5-S357A myo3Δ* mutant (figure 5.2). Second, these two kinases specifically interacted with the Myo5p motor head domain in a two-hybrid assay (figure 5.14). Finally, deletion of *MYO5* in a *pkh1-ts pkh2Δ* (Friant *et al*., 2001) yeast strain exacerbated the growth defect of this strain (see figure 5.14). Although deletion of *MYO5* alone does not cause any obvious phenotype for growth, it causes synthetic growth defects when combined with mutations in genes encoding proteins that closely cooperate with the myosins-I *in vivo* (Geli *et al*., 2000; Soulard *et al*., 2002). The slow-growth phenotype of the *myo5Δ pkh2Δ pkh1-ts* triple mutant therefore strongly suggests that Myo5p and the yeast kinases Pkh1p and Pkh2p cooperate *in vivo*.

Friant and co-workers have shown that Pkh1p and Pkh2p are activated by sphingoid bases and that overexpression of Pkh1p and Pkh2p suppresses the sphingoid base requirement in endocytosis (Friant *et al*., 2001). The fact that no sphingoid bases had been added to the *in*

vitro phosphorylation screen might also explain why the GST-Myo5-TEDSp was phosphorylated to only a low extent by Pkh2p (figure 5.13). Together with the data from deHart *et al*. (2002) and Friant *et al*. (2001), the findings from this study might therefore indicate that type I myosins are downstream targets of sphingoid base signalling through the kinases Pkh1p and Pkh2p. However, expression of Myo5-S357Ep was not able to overcome the requirement for these kinases in endocytosis and actin cytoskeleton polarization (figure 5.15). One possible explanation is that the myosins-I are not the only Pkh1p and Pkh2p targets for endocytosis, just like the myosins-I are probably not the only PAK targets for actin polarization. Indeed two other downstream targets of Pkh1p and Pkh2p, Pkc1p and Ypk1p, have been shown to be required for endocytosis (Friant *et al*., 2000; deHart *et al*., 2002). Moreover, Pkc1p was able to partially suppress the defect of missing sphingoid bases in this process (Friant *et al*., 2000).

Pkh1p and Pkh2p are homologues of the mammalian 3-phosphoinositide-dependent kinase PDK1 (Casamayor *et al*., 1999). This protein kinase is known to activate a number of downstream kinases that bear the consensus motif **Thr**-Phe-Cys-Gly-Thr-X-Glu-Tyr, where the first Thr is the phosphorylatable residue and X is any amino acid. These kinases include members of the protein kinase C (*PKC*), protein kinase A (*PKA*) and the serum and glucocorticoid-inducible kinase (*SGK*) families (Dutil *et al*., 1998; Egawa *et al*., 2002; Moore *et al*., 2002; Perrotti *et al*., 2001). *In vitro*, Pkh1p and Pkh2p are able to phosphorylate the yeast PKC and SGK homologues (Pkc1p and Ypk1p, Ypk2p, respectively) (Casamayor *et al*., 1999; deHart *et al*., 2002; Friant *et al*., 2001). Myo3p and Myo5p do not bear the PDK1 consensus motif and therefore, these molecular motors are unlikely to be direct targets of Pkh1p or Pkh2p. Instead, another kinase or kinases that co-purify with Pkh2p in the chip analysis might perform this function. This would also explain the low level of phosphorylation that could be observed in the *in vitro* screen (figure 5.13). It is important to note that the yeast kinases used in the biochemical screen are expressed and extracted from yeast under non-denaturing conditions and that they are only partially purified (Zhu *et al*., 2000). Possible candidates as intermediates in the sphingoid-base signalling pathway to the myosins-I are Pkc1p and Ypk1p because both are phosphorylated by Pkh1p and Pkh2p and both have been implicated in the endocytic uptake (deHart *et al*., 2002; Friant *et al*., 2001). However, Pkc1p or Ypk1p were not found in the biochemical screen and they did not seem to interact with the Myo5p head in the two-hybrid assay (figure 5.14). Further, attempts to phosphorylate the Myo5p TEDS sites using purified Pkc1p or Ypk1p have not succeeded so far (data not shown).

Another interesting possibility is that the yeast homologue of PKA might be involved in the process. Three genes encode catalytic subunits homologous to PKA in yeast, Tpk1p, Tpk2p and Tpk3p, and one gene encodes a regulatory subunit, Bcy1p (for review see Thevelein and de Winde, 1999). One of the catalytic subunits, Tpk1p, was indeed found to efficiently phosphorylate the GST-Myo5-TEDSp fusion protein in the chip assay (figure 5.13). Moreover, the sequence around the Myo5p TEDS site (Lys-Arg-Gly-**Ser**-Val) matches both of two alternative published requirements of PKA substrates, a lysine at position –3 and at least two N-terminal basic residues (Denis *et al*., 1991). Consistent with the idea that the yeast PKA might be a downstream effector of Pkh1p and Pkh2p, Tpk3p was found in complex with Pkh1p (Ho *et al*., 2002).

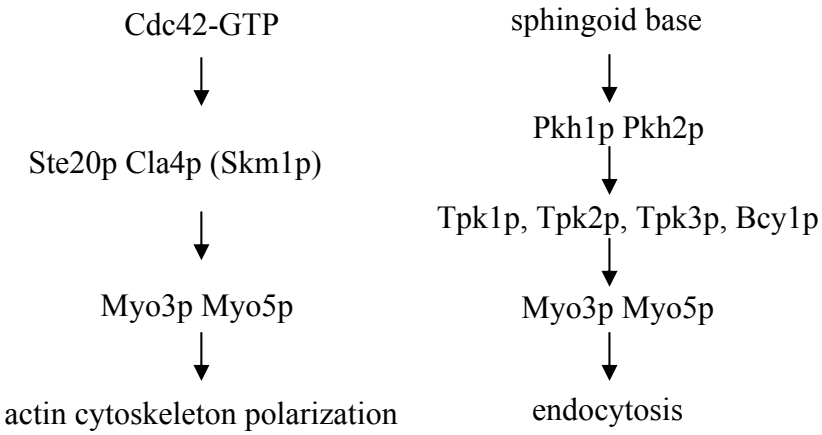

Figure 6.1: A new putative pathway for TEDS site phosphorylation and activation of myosins-I in endocytosis

PAKs and Cdc42p are required for actin cytoskeleton polarization, but not for endocytosis, while TEDS site phosphorylation was found to be necessary for both processes. Thus, Cdc42p and PAKs might be upstream factors for the type I myosins in their common function to polarize the actin cytoskeleton, but not in their function in endocytosis. The findings that Myo5p interacts *in vivo* and *in vitro* with the kinases Pkh1p and Pkh2p indicates that those might be the upstream factors for the myosin-I function in endocytosis. However, since the sequence around the Myo5p TEDS site does not match the phosphorylation consensus defined for the Pkh1p and Pkh2p human homologue PDK1, it seems unlikely that these kinases phosphorylate Myo5p directly. Rather, data indicate that the yeast PKA, which consists of the catalytic subunits Tpk1p, Tpk2p and Tpk3p and of the regulatory subunit Bcy1p, might fulfil this function. Consistently, Tpk1p was found to efficiently phosphorylate a GST-Myo5-TEDSp protein in the *in vitro* kinase screen.

If the yeast PKA activates the type I myosins for their function in endocytosis, PKA itself should be required for this process. So far, a role in endocytosis of the catalytic or regulatory subunits has not been published. However, some evidence supports a role of cAMP signalling in internalization, since the protein Srv2p, which binds to the adenylate cyclase and is required for cAMP signalling (Freeman *et al*., 1996; Yu *et al*., 1999), has been found to be involved in

endocytosis (Wesp *et al*., 1997). Most interestingly, Srv2p has also been implicated in the regulation of actin polymerization (Freeman *et al*., 1996).

Taken together, the data from this study suggest a new hypothesis outlined in figure 6.1. As discussed above, it is possible that PAKs phosphorylate the type I myosins for their function in actin cytoskeleton polarization. However, the activation of type I myosins for their function in endocytosis might be mediated by a second pathway, in which sphingoid bases activate the yeast kinases Pkh1p and Pkh2p to phosphorylate the yeast PKA homologue (Tpk1p, Tpk2p, Tpk3p, Bcy1p), which in turn would activate type I myosins (figure 6.1).

Further analysis of this hypothesis will now require to determine whether the yeast PKA is required for endocytosis and if this kinase indeed phosphorylates the type I myosins at their TEDS site.

6.1.4 Endocytosis is not dependent on actin cytoskeleton polarization

The fact that mutants defective in endocytosis display a depolarized actin cytoskeleton led scientist to suggest that functional uptake requires a polarized actin cytoskeleton (for review see Munn, 2001; Pruyne and Bretscher, 2000b). For this reason, Goodson and co-workers argued that the defect in endocytosis in a type I myosin double mutant is secondary to the defect in actin cytoskeleton polarization (Goodson *et al*., 1996). However, recent data already indicated that this were not the case. Mutants that are defective in actin cytoskeleton polarization, such as mutants of the actin-monomer-binding protein profilin and the type V myosin Myo2p, did not display a defect in the uptake step of endocytosis (for review see Munn, 2001). Moreover, wild-type yeast cells display a depolarized actin cytoskeleton after shift to 37°C, while endocytosis is not affected at this temperature (figures 5.9 and 5.11).

Data from this study now provide clear evidence that actin cytoskeleton polarization is not necessary for functional uptake at the plasma membrane in yeast. PAK (*ste20Δ skm1Δ cla4-as3*) mutant cells displayed a completely depolarized actin cytoskeleton after treatment with the Cla4-as3p kinase inhibitor 1NM-PP1, but did not display any defect in endocytosis under the same conditions (figure 5.10).

6.2 Casein kinase II (CKII) phosphorylates Myo5p at serine 1205 and possibly regulates actin assembly and endocytosis

The data presented in the second part of this study provide evidence that Myo5p is phosphorylated by the yeast casein kinase II (CKII) at serine 1205, which is located directly N-terminal to the acidic domain in the myosin tail, which binds to and activates the actin

nucleating Arp2/3 complex. Indeed, *in vitro* experiments revealed that phosphorylation of this residue might negatively regulate actin assembly. As described in the Introduction, actin cytoskeleton polarization and endocytosis seem to require Arp2/3 complex-mediated actin assembly. Consistent with a possible role of CKII in myosin-I-induced actin assembly, *in vivo* data indicate that CKII is required for proper maintenance of actin cytoskeleton polarity (Rethinaswamy *et al*., 1998) and preliminary data indicate that endocytosis is negatively regulated by one of the catalytic subunits of CKII *in vivo*. However, Myo5p tail phosphorylation at serine 1205 did not influence endocytosis. It is possible that other proteins that are involved in endocytosis are redundant CKII targets. Alternatively, myosin tail phosphorylation might regulate processes other than endocytosis and actin cytoskeleton polarization. In this case, the relevant CKII target(s) for its function in endocytosis would not be the myosins-I.

6.2.1 At least three serine residues of Myo5p are phosphorylated

The finding that the Myo5-S357Ap mutant was phosphorylated to a similar extent as wild-type Myo5p, indicated that there were phosphorylation sites other than the TEDS site in Myo5p (figure 5.12). Together with data that myosin-I-induced actin polymerization is regulated by phosphorylation/dephosphorylation (figures 5.16 and 5.17), this finding led to the identification of serine 1205 as a phosphorylation site for the yeast casein kinase II (CKII) (figure 6.2).

In vivo phosphorylation experiments with a Myo5-S357Ap truncation carrying the head, neck and TH1 domain additionally revealed that another phosphorylation site is located within these domains (data not shown). Consistent with this, Ficarro and co-workers identified Myo5p serine 777 as a phosphorylation site using mass spectrometry (Ficarro *et al*., 2002). This residue is located at the C-terminal end of the neck domain (figure 6.2), and might thus be responsible for the *in vivo* phosphorylation signal observed in the mentioned experiment within this study.

The fact that only this residue was identified in the mass spectrometry approach probably indicates that less Myo5p is phosphorylated at the other two phosphorylation sites at steady state, as has already been discussed for the TEDS site serine (see above).

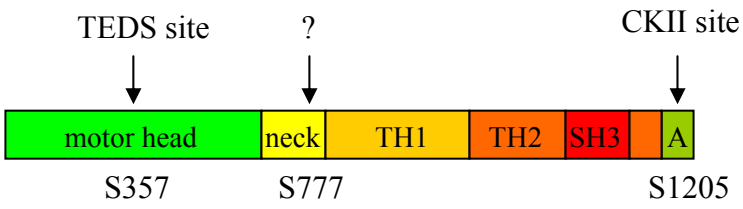

Figure 6.2: Myo5p phosphorylation sites

The TEDS site serine 357 of the yeast type I myosins was known to be a phosphorylation site. In this study, serine 1205 could be identified as an additional phosphorylation site in the Myo5p protein. Moreover, phosphopeptide mass spectrometry data have shown that serine 777 is phosphorylated as well.

6.2.2 CKII phosphorylates Myo5p serine 1205 *in vitro* and *in vivo*

This work provides robust *in vitro* evidence that yeast CKII phosphorylates serine 1205 in the Myo5p protein. Incubation of a GST-Myo5-Cp, which includes the TH2, SH3 and acidic domain of Myo5p fused in frame to GST, with wild-type yeast extract in the presence of heparin, an inhibitor of CKII, led to a significant reduction of phosphorylation (figure 5.21). Moreover, incubation with an extract from a temperature-sensitive CKII mutant strain (*cka1Δ cka2-ts*) led to the disappearance of the phosphorylation signal, while purified human CKII phosphorylated the GST-Myo5-Cp protein with high efficiency (figure 5.21). Mutation of serine 1205 into alanine or glutamate, respectively, abolished the *in vitro* phosphorylation of the Myo5p tail (figure 5.19). *In vivo* radiolabelling followed by immunoprecipitation and autoradiography gave additional support for the *in vivo* occurrence of this phosphorylation event (figure 5.20). Finally, two-hybrid analysis indicated that the regulatory subunits of CKII, Ckb1p and Ckb2p, are able to bind to the tail domain of Myo5p, suggesting the CKII and the type I myosin Myo5p might interact in the cells (figure 5.22).

6.2.3 Possible functions of Myo5p tail phosphorylation

6.2.3.1 *In vitro* actin polymerization is impaired in a *myo5-S1205E* mutant

In vitro data indicated that myosin-I induced actin polymerization was regulated by phosphorylation (figure 5.17). Moreover, these data implied that a kinase negatively influenced this process since incubation of GST-Myo5-Cp with wild-type yeast extract and phosphatase inhibitors led to a significant reduction in actin assembly (figure 5.17).

Since Myo5p serine 1205 was found to be phosphorylated *in vitro*, the possibility arose that it was this phosphorylation event that negatively affected actin polymerization, particularly since

serine 1205 is located in the acidic amino acid stretch that activates actin polymerization through the Arp2/3 complex (Evangelista *et al*., 2000; Geli *et al*., 2000; Lechler *et al*., 2000; see Introduction for more detail). Intriguingly, mutation of serine 1205 into glutamate, which mimics phosphorylation through the charge, significantly decreased the number of actin foci that appeared at the surface of GST-Myo5-S1205E-Cp-coated glutathione Sepharose beads (figure 5.23). Interestingly, incubation of glutathione Sepharose beads carrying the GST-Myo5-S1205E-Cp protein with a cell extract from a *myo5-S1205E myo3Δ* strain led to a further decrease in number of actin *foci* on the surface of the beads (data not shown). This result might indicate that not only the Myo5p tail fraction bound to the beads participates in the Arp2/3-dependent actin assembly, but that the cytosolic Myo5p is also recruited to the beads and that it might contribute to the process.

6.2.3.2 CKII might negatively regulate endocytosis by inhibiting actin polymerization

Preliminary experiments indicated that deletion of *CKA2*, but not *CKA1*, causes a significant increase in endocytosis, implying that Cka2p negatively regulates this process (figure 5.24). Interestingly, it has been shown that despite their apparent functional redundancy (Padmanabha *et al*., 1990), *CKA1* and *CKA2* mutants affect different functions. Glover suggests that Cka1p is involved in cell cycle regulation, while Cka2p appears to participate in the regulation of actin polarization (Glover, 1998). These findings indicate that the latter catalytic subunit might indeed be able to regulate processes such as endocytosis, which require actin rearrangements.

Taking the data from the second part of this thesis together, it seems that the yeast CKII, and in particular the Cka2p catalytic subunit, phosphorylates the type I myosin Myo5p at serine 1205 in its tail domain. Since the second type I myosin, Myo3p, also contains a putative CKII phosphorylation site in its C-terminal end of the tail domain (serine 1256), it appears likely that CKII also phosphorylates this site. Analysis of the Myo5-S1205Ep mutant protein using the *in vitro* actin polymerization assay, suggests that CKII–mediated phosphorylation of the type I myosins negatively regulates myosin-I-induced actin polymerization. It, therefore, seems reasonable to hypothesize that the effect of the CKII mutant on endocytosis might be caused by a constitutive de-inhibition of the myosin-I-induced actin polymerization (figure 6.3). However, neither endocytosis nor actin cytoskeleton polarization were affected by mutations of the Myo5p serine 1205, neither to alanine (putative constitutively dephosphorylated, i.e. active state) nor to glutamate (putative constitutively phosphorylated, i.e. inactive state) (figure 5.25). As described in the Results, mutation of serine 1205 to alanine did not

significantly affect the *in vitro* polymerization assay either (figure 5.23). It is likely that full activation of actin polymerization and endocytosis requires simultaneous dephosphorylation of different CKII targets involved in endocytosis. Consistent with this, CKII sites are also found in other proteins that are required for endocytosis, e.g. Pan1p, the yeast Eps15 homologue (Wendland and Emr, 1998) and Las17p, the yeast WASP homologue (Winter *et al*., 1999) (figure 6.3). Although Las17p does not contain a perfect CKII phosphorylation site, it does display the most important requirement for CKII, i.e. an acidic residue at position +3 (Pinna, 1990).

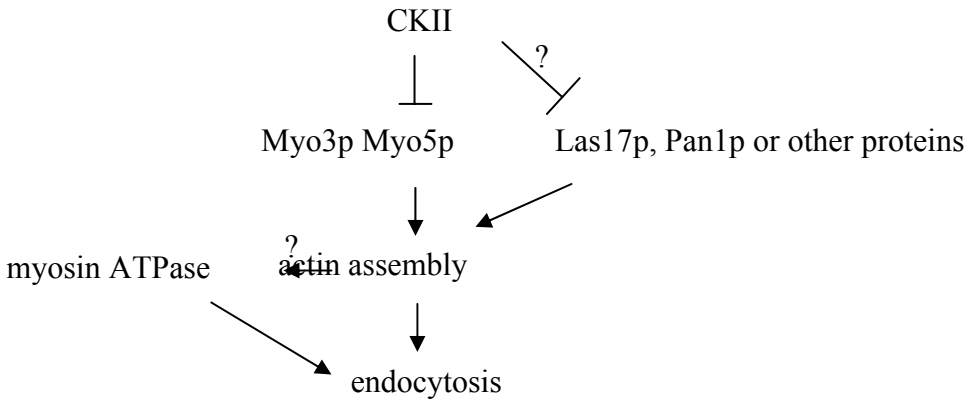

Figure 6.3: A putative pathway that leads to negative regulation of endocytosis in yeast

CKII phosphorylates the type I myosin Myo5p at serine 1205 in its tail domain. The second myosin-I, Myo3p, carries a putative CKII phosphorylation site at a similar position (serine 1256). Myosin-I-induced actin polymerization was inhibited in a Myo5p serine 1205 to glutamate mutant, indicating that CKII phosphorylation of the myosins-I might negatively regulate myosin-I-induced actin assembly, which has been shown to be required for endocytosis. Consistent with these findings and an inhibitory role of CKII in endocytosis, mutation of one catalytic CKII subunit seems to lead to faster internalization kinetics. However, mutation of the Myo5p serine into alanine (constitutive dephosphorylated state) did not lead to an obvious acceleration of endocytosis and mutation to glutamate (constitutive phosphorylated state) did not inhibit the process. It seems likely that other factors, e.g. Las17p, can compensate for the loss of activity of the *myo5-S1205E* mutant in actin assembly, and dephosphorylation of other CKII targets might be required for full activation of the process *in vivo* (see main text).

The finding that the acidic domains of the type I myosins and Las17p function redundantly (Evangelista *et al*., 2000) might explain why mutation of the myosin serine 1205 to glutamate in the acidic domain did not lead to any obvious phenotypic defect *in vivo*. In this context, it is important to mention that most *in vitro* assays are in general quite sensitive to lack of function mutations that might be compensated *in vivo* by more active redundant pathways. Further studies using site-directed mutagenesis of the CKII sites of the proteins involved in endocytosis might help to elucidate the precise function of these phosphorylation events and might yield additional data about the targets of CKII.

Finally, another possible explanation for the absence of obvious phenotypes in the Myo5p serine 1205 mutant strains is that tail phosphorylation regulates functions of the yeast type I myosins, which were not examined within this study. The relevant CKII target in endocytosis might then be different from the type I myosins. Recently, it has been demonstrated that Myo3p and Myo5p might be required for vacuolar membrane fusion (Eitzen *et al*., 2002). Moreover, evidence from mammalian cells indicates that type I myosins can function in the regulation of transcription (Dragovich *et al*., 2000), another function that is regulated by CKII (Hockman and Schultz, 1996). It might thus be these, or other, as yet unidentified functions of myosins-I that are regulated through Myo5p tail phosphorylation by CKII.

6.2.3.3 Myo5p tail phosphorylation: Regulation of the ATPase and/or oligomerization?

Myosin minifilaments have been observed for *Acanthamoeba castellanii* myosins-II (figure 6.4; Sinard and Pollard, 1989; Sinard *et al*., 1989), whose assembly seems to be regulated by tail phosphorylation (Baines and Korn, 1990; Collins *et al*., 1982; Ganguly *et al*., 1992). Additionally, data from *Dictyostelium discoideum* indicate that myosin-II tail phosphorylation inhibits filament formation by stabilizing a polymerization-incompetent bent dimer (Egelhoff *et al*., 1993; Pasternack and Racusen, 1989), which resembles the effect of light chain phosphorylation on vertebrate myosin-II (for review see Sellers and Goodson, 1995).

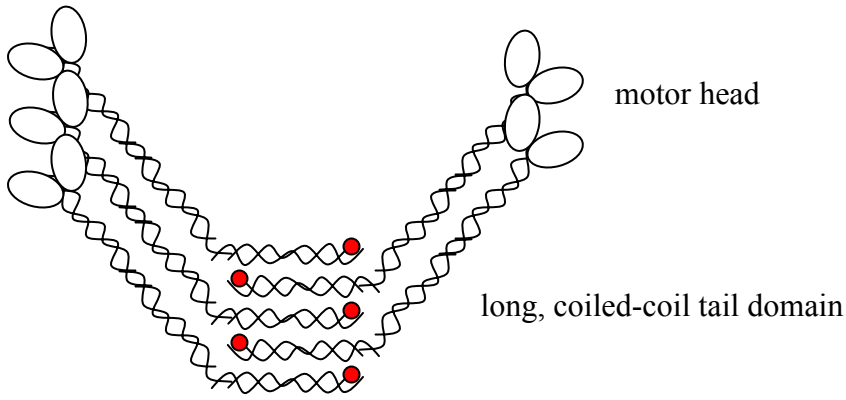

Figure 6.4: Myosin-II minifilaments

Data from type II myosins from *Acanthamoeba* and *Dictyostelium* indicate that tail phosphorylation (the location of the phosphorylation site is indicated in red) influences the assembly of these myosins into short filaments. Moreover, this phosphorylation appears to inhibit the motor ATPase activity.

An interesting, but speculative possibility of the function of the serine 1205 phosphorylation site arose from the finding that much more Myo5-S1205Ap than Myo5p was immunoprecipitated when taking the same amount of cells for the yeast extract and the same amount of antibody (figure 5.24). Besides the possibility that the S1205A mutation somehow

affected epitope accessibility, it is tempting to speculate that phosphorylation at serine 1205 regulates polymerization of Myo5p.

This has to happen in a different way from that of *Acanthamoeba* myosin-II, since type I myosins do not contain a long coiled-coil tail domain. Oligomerization could possibly be mediated by local induction of actin polymerization and the following ongoing binding of myosins-I via their tails or even their head domains to the actin filaments.

Myosin catalytic activity and its regulation have been attributed solely to the head domains (for review see Sellers, 2000), but other data suggest that tail domains can be involved in regulating myosin activity. Phosphorylation of serine residues in the tails of *Dictyostelium* and *Acanthamoeba* type II myosins down-regulates their actin-dependent ATPase activity (Truong *et al*., 1992; Baines and Korn, 1990; Collins *et al*., 1982; Ganguly *et al*., 1992). The question arises how a phosphorylation event so far away from the head motor can influence its activity. Again, the answer might come from the formation of minifilaments, where the tail phosphorylation site comes close to the neck region of the neighboring myosin-II (figure 6.4). Data indicate that this tail phosphorylation influences the activity of the head by altering the conformation of the hinge region of the adjacent myosin molecule (for review see Brzeska and Korn, 1996; Kuznicki *et al*., 1983). This cooperative regulation is supported by the finding that phosphorylation does not affect the actomyosin activity of monomers, but only the activity of copolymers (Atkinson *et al*., 1989; Ganguly *et al*., 1992; Kuznicki *et al*., 1983).

It is possible that yeast type I myosin tail phosphorylation might also regulate the ATPase of these motors. One possibility is that the local increase in F-actin concentration, mediated by Arp2/3 activation through the myosin tail domains, could in turn directly activate the motor head actin-dependent myosin ATPase activity (figure 6.3).

6.3 Outlook

Data in this study indicated that the TEDS site of the type I myosin Myo5p head domain is phosphorylated not only by p21-activated kinases (PAKs). Other kinases seem to activate the myosins for their function in endocytosis. The yeast type I myosins have been found to interact genetically and physically, *in vitro* and *in vivo*, with the kinases Pkh1p and Pkh2p, which are putative PKA upstream factors. The yeast PKAs, encoded by *TPK1*, *TPK2*, *TPK3* and *BCY1* are likely candidates for myosin-I kinases, since the Myo5p TEDS site is a good PKA recognition site and Tpk1p was found to phosphorylate Myo5p in an *in vitro* screen. Further testing this hypothesis will now require the analysis of endocytosis in *tpk* mutant cells. If any mutant strain exhibits a defect in internalization that can be suppressed by expression of Myo5-

S357Ep, this would constitute strong *in vivo* evidence for a role of PKA as the TEDS site kinase that activates type I myosins for their endocytic function. To further investigate the signalling cascade proposed to control Myo5p function in endocytosis, it has to be biochemically characterized whether sphingoid base-activated Pkh1p and/or Pkh2p activate the yeast PKA. Finally, since results suggest that activation of myosin-I occurs locally, the cellular localization of the myosins-I with the putative TEDS site kinases and the upstream activators has to be investigated in detail. If the hypothesis is correct, co-localization of myosin-I with their activators might serve to define the sites of endocytosis and might help to understand the role of the myosins-I both in endocytosis and in the polarization of the actin cytoskeleton

Another important finding in this study was that the TEDS site is not the only phosphorylation site in Myo5p. Rather, at least two more phosphorylation sites can be found. One of them, serine 1205 in the Myo5p tail domain, appears to be phosphorylated by the yeast casein kinase II (CKII). Our data suggest that CKII-mediated phosphorylation of myosin-I might inhibit Arp2/3-dependent actin polymerization and thereby inhibit endocytosis. The next goal is to further characterize the role of CKII as a negative regulator of actin polymerization *in vitro*, i.e. to investigate whether CKII mutant cytosols sustain the formation of a greater number of actin patches on the Myo5p coated bead and whether addition of purified CKII to the assay inhibits the process. Further, *in vitro* phosphorylation assays with wild-type and CKII mutant cytosols might help to identify other putative CKII targets required for the formation of actin patches and endocytosis. The identification of other putative targets of CKII involved in endocytosis might help to elucidate the *in vivo* role of CKII in modulating the endocytic uptake. Additionally, it will be interesting to define the phosphatase that counteracts CKII phosphorylation.

7 Materials and Methods

7.1 Yeast strains and genetic techniques

Unless otherwise mentioned, strains were grown in complete yeast peptone dextrose media (YPD) or, if selection was required, in appropriate synthetic dextrose minimal media (SDC) (Sherman, 1991). Complete media contained 1 % yeast extract (Difco, Heidelberg), 2 % peptone (Difco, Heidelberg) and 2 % glucose (Fluka, Steinheim). Synthetic minimal media consisted of 2 % glucose (Fluka, Steinheim), 0.67 % yeast nitrogen base (Difco, Heidelberg) and 0.075 % of CSM (complete synthetic mix; Qbiogene, Heidelberg), which contains all required amino acids, purine- and pyrimidine-bases except for those required for selection of auxotrophic markers. SDC-Ura media, for instance, contained all described components except for uracil. The concentrations of all amino acids, purin- and pyrimidin-bases used in CSM were: 10 mg/l adenine, 50 mg/l L-arginine, 80 mg/l L-aspartate, 20 mg/l L-histidine-HCl, 50 mg/l L-leucin, 50 mg/l L-lysine, 20 mg/l L-methionine, 50 mg/l L-phenylalanine, 100 mg/l L-threonine, 50 mg/l tryptophane, 50 mg/l L-tyrosine, 20 mg/l uracil and 140 mg/l valine. Solid media additionally contained 2 % agar (Fluka, Steinheim).

Sporulation, tetrad dissection and scoring of genetic markers were performed as described (Sherman *et al*., 1974). Briefly, in order to obtain diploid yeast cells, haploid cells of opposite mating types, *Mat***a** and *Mat*α, were mixed on YPD plates and incubated for approximately 12 hours. Subsequently, diploid cells were selected on appropriate minimal media (SDC lacking all amino acids, purine- or pyrimidine-bases that could be synthesized by the diploid but not by the haploid yeast cells). For sporulation, diploid cells were grown for 1 day on complete solid media and subsequently transferred to sporulation media (0.022% raffinose, 3g/l potassium acetate). The spores were separated (dissected) under a tetrad microscope (Singer Instruments, Somerset, England) and allowed to germinate and grow on complete media.

The mating type of haploid cells was tested by plating the corresponding yeast either with *Mat***a** or *Mat*α tester strains bearing a *his1* mutation (not present in any other laboratory strain) on minimal media lacking all amino acids, purine- or pyrimidine-bases. Only yeast of mating type opposite to the testers were able to produce diploids that grew on minimal media. In order to obtain spontaneous *ura3* mutant cells, cells were selected on minimal media containing 5'-FOA (5'-Fluoroorotic acid). The enzyme encoded by the wild type *URA3* gene, orotidine-5'-phosphate-decarboxylase, produces 5'-fluoro-uracil in the presence of 5'-FOA. This product is

toxic for yeast cells (Boeke *et al*., 1987). Therefore, cells that survive on 5'-FOA containing plates do not synthesize this enzyme, implicating that the encoding gene is mutated.

For gene disruption by homologous recombination, either PCR fragments were synthesized, which contained a vegetative yeast marker and flanked by DNA sequences homologous to the gene of interest (about 40 nucleotides upstream and downstream of the region to be disrupted) or integration cassettes were used. For integration of point mutations at the corresponding locus, integration plasmids were used. These plasmids do not carry autonomous replication sequences (*ARS*/*CEN* or 2μ).

The knockout of *BAR1*, a secreted protease that cleaves the α-factor and interferes with the α-factor uptake (see below), was tested by a plate assay. A lawn of a yeast strain hyper-sensitive to α-factor (*Mat***a** *ssa1 ssa2*) was plated on YPD. A line of a *Mat*α strain was then streaked in the middle of this plate. Upon 1 day incubation at 30°C, secretion of α-factor by the *Mat*α strain arrested growth of the *Mat***a** *ssa1 ssa2* cells therefore producing an halo in the middle of the plate. To identify *bar1* mutant yeast, the strains to be tested were streaked perpendicular to the line of the *Mat*α strain. Wild type *BAR1* yeasts secrete the protease and degraded the α-factor, thus allowing the lawn of the α-factor hyper-sensitive strain to grow closer to the *Mat*α strain middle line. If *BAR1* was knocked out, the secreted pheromone was not cleaved and the *Mat***a** *ssa1 ssa2* strain was not able to grow closer to the *Mat*α strain.

Transformation of yeast was accomplished by the lithium acetate method (Ito *et al*., 1983).

7.1.1 Yeast strains

The yeast strains used in this study are listed in Table I. Not previously published strains were generated as follows.

SCMIG567, **568 and 569**

In order to obtain yeast cells that express similar amounts of wild type or mutant Myo5p, the corresponding genes were integrated in the yeast genome. For this purpose, plasmids pIN*MYO5*, pIN*myo5-S357A* and pIN*myo5-S357E* were digested with *Sna*BI, which cuts once in the promotor region. These linearized plasmids were transformed into SCMIG51 (*myo5Δ*) and selected on SDC-Ura-Trp, allowing integration of the genes in the *MYO5* locus by homologous recombination (figure 7.1). Transformants were then tested for wild-type-like expression of *MYO5* or *myo5* mutants by Western blot and good candidates were finally crossed to SCMIG52 (*myo3Δ*) and selected on SDC-Ura-Trp-His. The diploids were sporulated, tetrads dissected and spores scored for the appropriate markers indicating co-segregation of the integrated *MYO5* (SCMIG567), *myo5-S357A* (SCMIG568) or *myo5-S357E*

(SCMIG569) with a *myo3* deletion (*myo3Δ*) (figure 7.1). Co-segregation of the *URA3* marker with the *TRP1* marker indicated that *MYO5* or the *myo5* mutants were integrated in the *MYO5* locus.

SCMIG582, 583 and 584

SCMIG51 (*myo5Δ*) was transformed with plasmids p33*MYO5-HA*$_3$, p33*myo5-S357A-HA*$_3$ and p33*myo5-S357E-HA*$_3$, respectively, and selected on SDC-Ura. Transformants were crossed to SCMIG52 (*myo3Δ*) and selected on SDC-Ura-Trp-His, diploids were sporulated and spores examined for the appropriate markers indicating the presence of either p33*MYO5-HA*$_3$ (SCMIG582), p33*myo5-S357A-HA*$_3$ (SCMIG583) or p33*myo5-S357E-HA*$_3$ (SCMIG584) in a *myo3Δ myo5Δ* background.

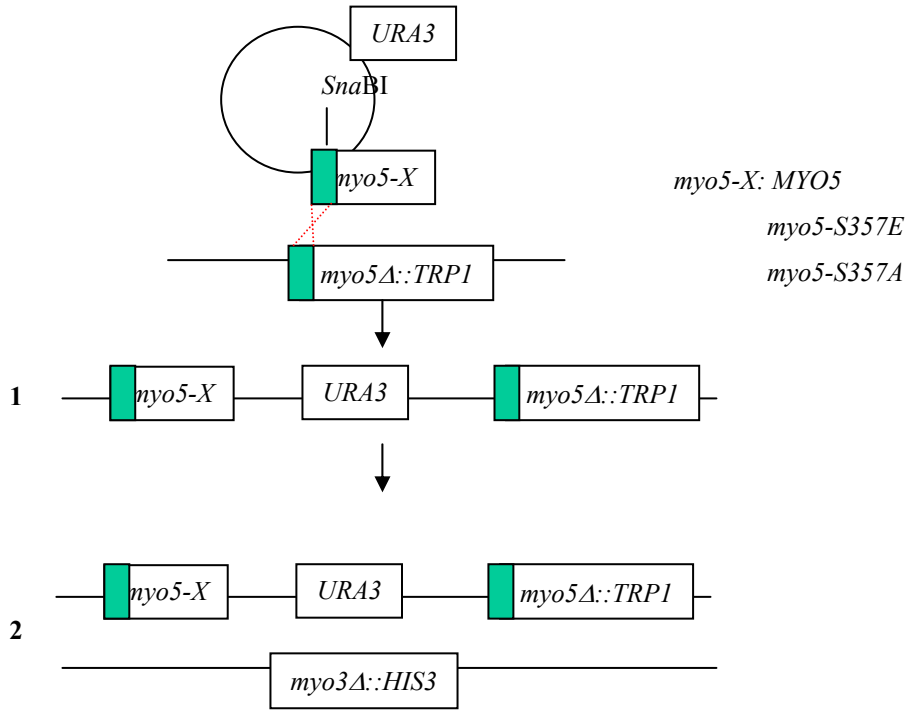

Figure 7.1: Construction of wild-type *MYO5* or *myo5* mutants in a *myo3Δ* background

pIN*MYO5*, pIN*myo5-S357A* and pIN*myo5-S357E* were cut with *Sna*BI and transformed into a *myo5Δ* strain. The region of homology (green) with a part of the endogenous *MYO5* promoter allowed integration at the *MYO5* locus (**1**). Crossing the resulting strains to a *myo3Δ* strain (**2**) resulted in strains SCMIG567 (*MYO5 myo3Δ*), SCMIG568 (*myo5-S357A myo3Δ*) and SCMIG569 (*myo5-S357E myo3Δ*).

Table I. Yeast strains

Strain	Genotype	Reference
SCMIG19	*Mat***a** *his3 leu2 trp1 ura3 bar1*	Idrissi *et al*., 2002

(RH2881)		
SCMIG21	*Matα his3 leu2 trp1 ura3 bar1*	Dr. M.I.Geli
SCMIG50	*Mat***a** *ade2 his3 leu2 trp1 ura3 bar1*	Geli *et al*., 1998
(RH3975)		
SCMIG51	*Mat***a** *ade2 his3 leu2 trp1 ura3 bar1 myo5Δ::TRP1*	Geli *et al*., 1998
(RH3976)		
SCMIG52	*Matα his3 leu2 trp1 ura3 bar1 myo3Δ::HIS3*	Geli *et al*., 1998
(RH3977)		
SCMIG69	*Mat***a** *lcb1-100 (ts; end8-1) leu2 ura3 his4 bar1*	Munn and Riezman, 1994
(RH2607)		
SCMIG217	*Matα cla4Δ::LEU2 ade2 his3 leu2 trp1 ura3*	Dr. M.I.Geli
SCMIG276	*Matα ade2 his3 leu2 lys2 trp1 ura3 bar1 myo5Δ::TRP1*	Dr. M.I.Geli
SCMIG567	*Mat***a** *his3 leu2 trp1 ura3 bar1 myo5Δ::TRP1 myo3Δ::HIS3 MYO5::URA3*	this study
SCMIG568	*Mat***a** *ade2 his3 leu2 trp1 ura3 bar1 myo5Δ::TRP1 myo3Δ::HIS3 myo5-S357A::URA3*	this study
SCMIG569	*Mat***a** *ade2 his3 leu2 trp1 ura3 bar1 myo5Δ::TRP1 myo3Δ::HIS3 myo5-S357E::URA3*	this study
SCMIG582	*Matα ura3 his3 trp1 leu2 bar1 ade2 myo5Δ::TRP1 myo3Δ::HIS3 pMYO5HA$_3$ (CEN URA3)*	this study
SCMIG583	*Matα ura3 his3 trp1 leu2 bar1 ade2 myo5Δ::TRP1 myo3Δ::HIS3 pmyo5-S357AHA$_3$ (CEN URA3)*	this study
SCMIG584	*Mat***a** *ura3 his3 trp1 leu2 bar1 ade2 myo5Δ::TRP1 myo3Δ::HIS3 pmyo5-S357EHA$_3$ (CEN URA3)*	this study
SCMIG271	*Matα ura3 lys2 trp1 his3 leu2 ade2 cdc25-2*	Aronheim and Karin, 2000
RH1987	*Mat***a** *ura3 trp1 ade2 his3 leu2 bar1 can1 ste20Δ::URA3*	Dr. H.Riezman
SCMIG574	*Mat***a** *ura3 trp1 ade2 his3 leu2 bar1 can1 ste20Δ skm1Δ::TRP1 cla4-H685Y::HIS3 (ts)*	this study
SCMIG618	*Mat***a** *CDC42::LEU2 ura3 leu2 his3 lys2 bar1Δ ::URA3*	this study
SCMIG619	*Mat***a** *cdc42-1(ts) ura3 his3 lys2 bar1Δ::URA3*	this study
SCMIG623	*Mat***a** *cdc42-123(ts)::LEU2 ura3 leu2 his3 lys2 bar1Δ ::URA3*	this study
SCMIG624	*Mat***a** *cdc42-129(ts,cs)::LEU2 ura3 leu2 his3 lys2 bar1Δ ::URA3*	this study
SCMIG625	*Mat***a** *cdc42-118 (ts)::LEU2 ura3 leu2 his3 lys2 bar1Δ ::URA3*	this study
MS883	*Matα ade2 leu2 his3 trp1 ura3 ste20Δ::kanMX cla4Δ::LEU2 skm1Δ::HIS3* p*cla4-as3 (CEN TRP1)*	Dr. M. Peter
SCMIG586	*Mat***a** *ade2 leu2 his3 trp1 ura3 bar1Δ ste20Δ::kanMX cla4Δ::LEU2 skm1Δ::HIS3* p*cla4-as3 (CEN TRP1)*	this study
SCMIG588	*Mat***a** *ade2 leu2 his3 trp1 ura3 bar1Δ ste20Δ::kanMX cla4Δ::LEU2*	

	skm1Δ::HIS3 p*CLA4 (CEN TRP1)*	this study
SCMIG603	*Mat***a** *leu2 ura3 his2 ade1 trp1 bar1*	this study
SCMIG604	*Mat***a** *ade1 his2 trp1 leu2 ura3 bar1 pkh2Δ::LEU2 pkh1-ts*	this study
SCMIG605	*Mat***a** *ade1 trp1 leu2 ura3 bar1 myo5Δ::TRP1 pkh2Δ::LEU2 pkh1-ts*	this study
EGY48	*Mat*α *ura3 leu2 his3 trp1*	Gyuris *et al*., 1993
SCMIG590	*Mat***a** *his3 leu2 trp1 ura3 bar1 myo3Δ::HIS3 myo5Δ::TRP1 pMYO5 (CEN URA3)*	this study
SCMIG591	*Mat***a** *his3 leu2 trp1 ura3 bar1 myo3Δ::HIS3 myo5Δ::TRP1 pmyo5-S1205A (CEN URA3)*	this study
SCMIG592	*Mat***a** *his3 leu2 trp1 ura3 bar1 myo3Δ::HIS3 myo5Δ::TRP1 pmyo5-S1205E (CEN URA3)*	this study
SCMIG629	*Mat***a** *lys2 his3 leu2 ura3 cka1Δ::HIS3 cka2Δ:: TRP1 pcka2-13 (LEU2, CEN)*	this study
SCMIG549	*Mat***a** *ade2 lys2 his3 leu2 ura3 cka1D::HIS3 cka2Δ:: TRP1 pCKA2 (LEU2, CEN) bar1::URA3*	this study
SCMIG553	*Mat***a** *ade2 lys2 his3 leu2 ura3 ura3 cka1Δ::HIS3 cka2Δ:: TRP1 pCKA1 (LEU2, CEN) bar1Δ::URA3*	this study

SCMIG574

A PCR-generated *SKM1* knockout cassette containing a yeast marker (*TRP1*) and additional flanking sequences of about 40 nucleotides corresponding to the upstream and downstream sequences of the fragment to be deleted, was generated using the primers ko-skm1-F and ko-skm1-R and plasmid p*skm1Δ::TRP1* as template. This PCR fragment was transformed into RH1987 (kindly provided by Dr. H. Riezman). Transformants were selected on SDC-Trp to recover yeast cells were the chromosomal copy of SKM1 was substituted by the *TRP1* marker by homologous recombination. The knockout was confirmed by PCR (primers 20LU1 and PAKD1) using the genomic DNA of the strain as a template. Subsequently, a PCR fragment containing the 3' end of *CLA4* including a His685Tyr (H685Y) mutation together with a wild type *HIS3* gene was created. The 3' end of *cla4-H685Y*, encoding the amino acids 522 until the STOP, was amplified from p*cla4-H685Y* using the primers Cla4.1619.D and Cla4.2529.U. The *HIS3* gene flanked by 40 nucleotides immediately upstream and downstream of the stop codon of *CLA4* was amplified from plasmid YDp-H with the primers Cla4.2509.YDp.D and Cla4.2579.YDp.U. The two resulting PCR fragments were used as templates for a subsequent PCR reaction with the Cla4.1619.D and Cla4.2579.YDp.U primers. This reaction yielded a hybrid DNA fragment encoding amino acids 540 to 843 of the *Cla4-H685Yp* mutant, the *HIS3* marker and 40 nucleotides downstream of the *CLA4* stop codon (figure 7.2). This DNA was

transformed into the *ste20Δ skm1Δ* strain, cells were selected on SDC-His-Trp-Ura in order to select the triple temperature-sensitive PAK mutant (figure 7.2). Proper insertion of the *cla4-H685Y* mutant in the *CLA4* locus was tested by crossing the transformants to SCMIG217 (*cla4Δ::LEU2*) and scoring for complete segregation of the *LEU2* and *HIS3* markers. All putative triple PAK mutant exhibited a strong temperature-sensitivity phenotype (inability to grow at 37°C).

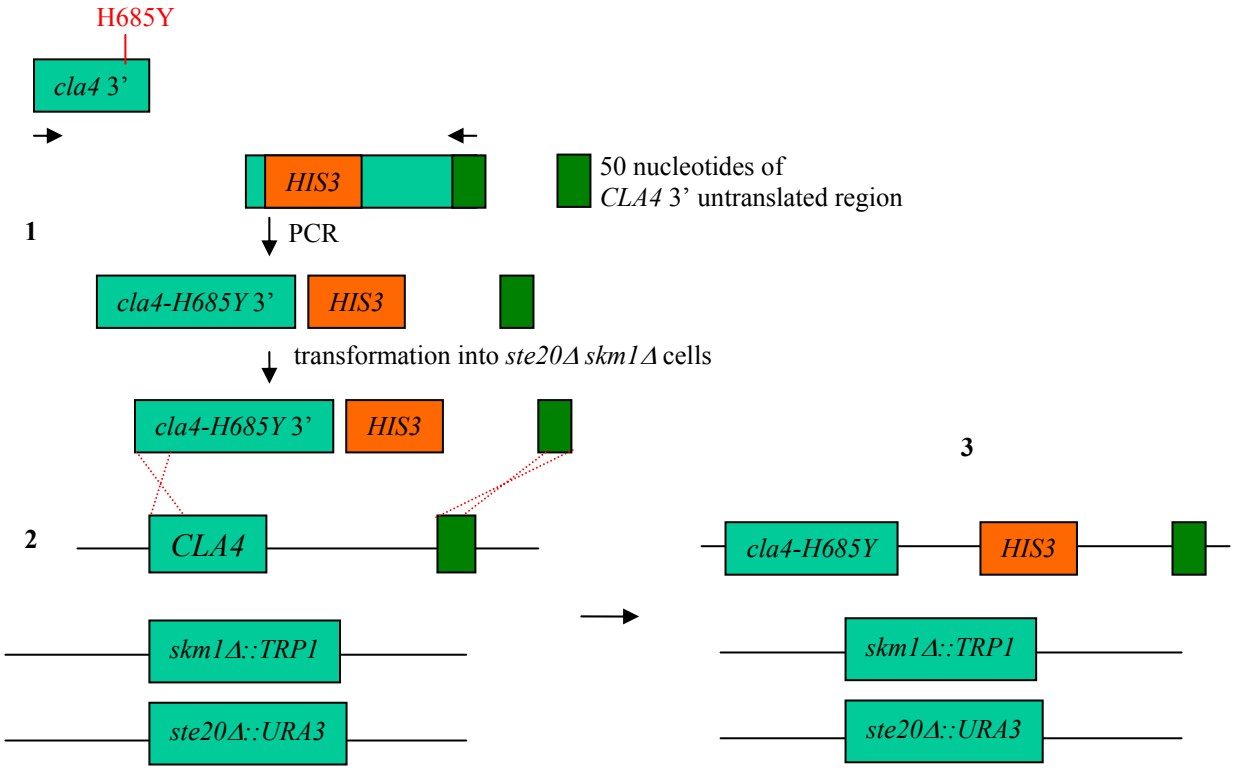

Figure 7.2: Construction of the temperature-sensitive PAK kinase mutant

The chromosomal copy of *SKM1* was substituted by the *TRP1* marker in a *ste20Δ::URA3* strain. A DNA fragment that carried the 3' end of *cla4-H685Y* containing the histidine to tyrosine codon substitution, and the *HIS3* gene after the *CLA4* stop codon, was amplified by a double PCR strategy (1). The triple PAK mutant was obtained by transformation of this DNA into the *skm1Δ ste20Δ* double mutant (2) and subsequent homologous recombination with the endogenous *CLA4* locus, thereby substituting the wild-type 3' end of *CLA4* with the mutant allele (3).

SCMIG618, 619, 623, 624 and 625

The chromosomal copy of the protease *BAR1*, which cleaves the α-factor and therefore interferes with the α-factor uptake assay, was substituted by the *URA3* gene using a *bar1Δ::URA3* knockout cassette, which contains the *URA3* marker flanked by 600 bp of the region downstream and 700 bp of the region upstream of the *BAR1* Open Reading Frame (ORF), in strains DDY1300, DDY1302, DDY1326, DDY1336 and DDY1344 (kindly

provided by K.Kozminski and D.Drubin; Kozminski *et al*., 2000). For this purpose, plasmid pLH309 was digested with *Eco*RI and transformed into the DDY strains. Transformants were selected on SDC-Ura and the disruption of *BAR1* was tested by using a plate assay as described before.

SCMIG586

In order to obtain a yeast strain of the mating type **a**, MS883 (kindly provided by M.Peter) was transformed with plasmid B1377 carrying the *HO* endonuclease under the *GAL1* promoter. This endonuclease mediates the mating-type switch, from **α** to **a** or reverse, in wild-type yeast and it has been inactivated in most laboratory strains. Production of this enzyme was induced by addition of galactose to a final concentration of 2 %. After 2, 4 and 6 hours samples were taken and plated on complete media. Colonies were then replica plated on minimal media containing 5'-FOA for contra-selection of the *HO-URA3*-plasmid. The mating type of colonies growing on 5'-FOA –containing media was checked and the *BAR1* gene of a resulting *Mat***a** strain was then substituted by the *URA3* gene as described before. To select *ura3* cells again, the strain was grown in complete media to saturation and plated on 5'-FOA. Colonies were finally checked for all markers and for their sensitivity to the Cla4-as3p inhibitor 1NM-PP1 (Weiss *et al*., 2000).

SCMIG588

Plasmid p*cla4-as3::URA3* was transformed into strain SCMIG586. To facilitate loss of the original p*cla4-as3* (*TRP1*), transformants were grown to saturation in SDC-Ura liquid media, plated on SDC-Ura and individual colonies finally checked for their inability to grow on SDC-Trp. A *URA3 trp1* strain was then transformed with p*CLA4*, which contains the wild-type *CLA4* (*TRP1*) and selected on 5'-FOA-containing plates in order to contra-select p*cla4-as3::URA3*. Finally, the resulting strain was tested for its insensitivity to the Cla4-as3p inhibitor 1NM-PP1 (Weiss *et al*., 2000).

SCMIG603, 604 and 605

Strains SCMIG603 and SCMIG604 were constructed by selecting for *ura3* mutants of RH5411 and RH5412 (kindly provided by H. Riezman; Friant *et al*., 2001), respectively, on 5'-FOA-containing plates. SCMIG605 was generated by crossing SCMIG604 with SCMIG276. Diploids were selected on SDC-Leu-Trp, sporulated, tetrads were dissected and segregants were examined for the presence of the *LEU2* and *TRP1* markers and for their temperature-sensitivity to identify the triple mutant, *pkh1*-ts *pkh2Δ::LEU2 myo5Δ::TRP1*.

SCMIG590, 591 and 592

SCMIG51 (*myo5Δ*) was transformed with plasmids p33*MYO5*, p33*myo5-S1205A* or p33*myo5-S1205E* and cells were selected on SDC-Ura. Resulting transformants were crossed to SCMIG52 (*myo3Δ*), diploids were selected on SDC-His-Ura-Trp. Subsequently, tetrads were dissected and *URA3 TRP1 HIS3* markers were analysed to identify the *myo5Δ myo3Δ* segregants bearing p33*MYO5* (SCMIG590), p33*myo5-S1205A* (SCMIG591) or p33*myo5-S1205E* (SCMIG592).

SCMIG629

Since *in vitro* phosphorylations could only be performed with extracts of *ADE2* strains, YDH13 (kindly provided by C.V.C.Glover; Glover, 1998) was transformed with the *ADE2* gene and selected on SDC-Ade (to obtain *ADE2*: plasmid pASZ12 digested with *Eco*RI and *Bst*BI). The use of red *ade2* strains extracts led to degradation of the GST-Myo5-Cp fusion protein in the *in vitro* polymerization assay (see below).

SCMIG551 and 553

The chromosomal copy of the protease *BAR1* was substituted by the *URA3* gene (as described before) in strains YAR13 and YAR108, respectively (kindly provided by C.V.C. Glover; Rethinaswamy *et al*., 1998).

7.2 DNA techniques and plasmid construction

Plasmids used in this study and their relevant features are listed in table II, primers in table III.

7.2.1. Plasmids

Table II. Plasmids

Plasmid[*]	**Yeast marker**[#]	**Insert**[+]	**Reference**
YDp-H	*HIS3*	-	Berben *et al*., 1991
YDp-W	*TRP1*	-	Berben *et al*., 1991
YDp-U	*URA3*	-	Berben *et al*., 1991
pASZ12	*ADE2*	-	Stotz and Linder, 1990
pFA6A-3HA-HIS3MX6	*HIS3*	-	Longtine *et al*., 1998
YCplac33	*URA3*	-	Gietz and Sugino, 1988
p33*MYO5*	*URA3*	*MYO5*	Geli and Riezman, 1996
p33*myo5-S357A*	*URA3*	*myo5-S357A*	Dr. M.I.Geli
p33*myo5-S357E*	*URA3*	*myo5-S357E*	Dr. M.I.Geli
pIN*MYO5*	*URA3*	*MYO5*	Dr. M.I.Geli
pIN*myo5-S357A*	*URA3*	*myo5-S357A*	Dr. M.I.Geli
pIN*myo5-S357E*	*URA3*	*myo5-S357E*	Dr. M.I.Geli

p33*MYO5-HA$_3$*	*URA3*	*MYO5* + 3HA	Idrissi *et al*., 2002
p33*myo5-S357A-HA$_3$*	*URA3*	*myo5-S357A*+ 3HA	this study
p33*myo5-S357E-HA$_3$*	*URA3*	*myo5-S357E*+ 3HA	this study
pYES-5'SOS	*URA3*	*5'SOS*	Aronheim and Karin, 2000
pYX*5'SOS*	*LEU2*	*5'SOS*	this study
pYX*5'SOS-MYO5*	*LEU2*	*5'SOS-MYO5*	this study
pYX*5'SOS-myo5-S357A*	*LEU2*	*5'SOS-myo5-S357A*	this study
pYX*5'SOS-myo5-S357E*	*LEU2*	*5'SOS-myo5-S357E*	this study
p*skm1Δ::TRP1*	*TRP1*	*skm1Δ::TRP1*	Dr. M.I.Geli
p*cla4-H685Y*	*HIS3*	*cla4-H685Y (*aa 521-1090*)*	Dr. M.I.Geli
B1377	*URA3*	*HO*	Dr. H.-U. Moesch
pLH309	-	*bar1Δ::URA3* (*BAR1* k.o.)	Dr. L. Hicke
p*cla4-as3*	*TRP1*	*cla4-as3*	Weiss *et al*., 2000
p*cla4-as3::URA3*	*URA3*	*cla4-as3*	this study
p*CLA4*	*TRP1*	*CLA4*	this study
pGEX-5X-3	-	GST	Pharmacia
pGST-*myo5*-TEDS	-	GST-*MYO5* (aa 322-391)	this study
pEG202	*HIS3*	LexA	Gyuris *et al*., 1993
pEG202 *myo5*-H	*HIS3*	LexA-*MYO5* (aa 1-773)	this study
pRFM-1	*HIS3*	LexA-Bicoid	Gyuris *et al*., 1993
pJG4-5	*TRP1*	B42	Gyuris *et al*., 1993
pJG4-5*PKH1*	*TRP1*	B42-*PKH1*	this study
pJG4-5*PKH2*	*TRP1*	B42-*PKH2*	this study
pJG4-5*YPK1*	*TRP1*	B42-*YPK1*	this study
pJG4-5*YPK2*	*TRP1*	B42-*YPK2*	this study
pJG4-5*ARK1*	*TRP1*	B42-*ARK1*	this study
pJG4-5*PRK1*	*TRP1*	B42-*PRK1*	this study
pJG4-5*YCK1*	*TRP1*	B42-*YCK1*	this study
pJG4-5*YCK2*	*TRP1*	B42-*YCK2*	this study
pJG4-5*PKC1*	*TRP1*	B42-*PKC1*	this study
pJG4-5*CLA4*	*TRP1*	B42-*CLA4*	this study
pJG4-5*IRE1*	*TRP1*	B42-*IRE1*	this study
p33*ProtA-MYO5*	*URA3*	*MYO5*	Dr. F.Idrissi
p33*ProtA-MYO5-HA$_3$*	*URA3*	*ProtA-MYO5*+ 3HA	this study
p33*ProtA-myo5-S357A-HA$_3$*	*URA3*	*ProtA-myo5-S357A*+ 3HA	this study
p33*ProtA-myo5-S357A*-H-*HA$_3$*	*URA3*	*ProtA-myo5-S357A* (aa 2-700)+ 3HA	this study
p33*ProtA-MYO5*-H-*HA$_3$*	*URA3*	*ProtA-MYO5* (aa 2-700) + 3HA	this study

pGST*MYO5*-C	-	GST-*MYO5* (aa 982-1219)	Idrissi *et al*., 2002
pGST-(SH3,TH2c)	-	GST- *MYO5* (aa 1085-1219)	Geli *et al*., 2000
pGST-(TH2c)	-	GST- *MYO5* (aa 1142-1219)	Geli *et al*., 2000
pGST *myo5-S1205A*-C	-	*myo5-S1205A* (aa 982-1219)	this study
pGST *myo5-S1205E*-C	-	*myo5-S1205E* (aa 982-1219)	this study
p33*myo5-S1205A*	*URA3*	*myo5-S1205A*	this study
p33*myo5-S1205E*	*URA3*	*myo5-S1205E*	this study
pEG202*MYO5*-T	*HIS3*	LexA-*MYO5* (aa 757-1219)	Geli *et al*., 2000
pJG4-5*CKA1*	*TRP1*	B42-*CKA1*	this study
pJG4-5*CKA2*	*TRP1*	B42-*CKA2*	this study
pJG4-5*CKB1*	*TRP1*	B42-*CKB1*	this study
pJG4-5*CKB2*	*TRP1*	B42-*CKB2*	this study

*All plasmids listed in this table carry a bacterial *ori* and an Amp^R resistance gene.
#Plasmids of the pEG202 and pJG4-5 series are 2µ (multi-copy) plasmids. All other plasmids, which contain a yeast marker, except the pIN series, are *CEN* (low-copy) plasmids.

Not previously published plasmids were generated as follows.

pIN*MYO5*, pIN*myo5-S357A* and pIN*myo5-S357E* (constructed by Dr. M.I. Geli) are integration plasmids. They do not harbour replication sequences, and contain the genes *MYO5*, *myo5-S357A* and *myo5-S357E*, respectively. These plasmids were constructed from the Ycplac33 based plasmids p33*MYO5*, p33*myo5-S357A* and p33*myo5-S357E* bearing the wild type and mutant *MYO5*, by deleting the *ARS* and *CEN* sequences of YCplac33 contained in an *Nsi*I/*Spe*I fragment. p33*myo5-S357A* and p33*myo5-S357E* were constructed by introducing the corresponding mutation on p33*MYO5* using a double PCR strategy (constructed by Dr. M.I. Geli).

p33*myo5-S357A-HA$_3$* and p33*myo5-S357E-HA$_3$*, respectively, were obtained by replacing an *Sph*I/*BstE*II fragment of plasmids p33*myo5-S357A* or p33*myo5-S357E* containing part of the 3' end of the mutants, with an *Sph*I/*BstE*II fragment from plasmid p33*MYO5-HA$_3$*, which was constructed by Dr. M.I. Geli. This latter fragment contains the analogous 3' part of *MYO5* followed by 3 HA epitopes.

pYX5'SOS-*MYO5*, pYX5'SOS-*myo5-S357A* and pYX5'SOS-*myo5-S357E* were obtained by inserting *MYO5*, *myo5-S357A* or *myo5-S357E*, respectively, in frame downstream of 5'SOS into pYX5'SOS. *MYO5* or *myo5* mutants were amplified from plasmids p33*MYO5*, p33*myo5-S357A* and p33*myo5-S357E* using primers Myo5.1.ApaI.D and Myo5.3634.MluI.U. pYX5'SOS and PCR fragments were digested with *Apa*I and *Mlu*I and the PCR fragments subsequently ligated into pYX5'SOS. Plasmids pYX5'SOS and p5'SOS-*MYO5* were constructed by H. Groetsch.

p*cla4-as3* was recovered from the yeast strain MS883 (kindly provided by Dr. M. Peter) (see below).

p*CLA4* was obtained by homologous recombination in yeast. For that purpose, p*cla4as3* was digested with *Nsi*I to remove the DNA encoding amino acids 363 to 865, which contains the two mutations in *cla4-as3*. A DNA fragment encoding amino acids 344 to 890 of wild type Cla4p was amplified from wild-type genomic DNA by PCR using the primers Cla4-1031-D and Cla4-2669-U.. The *Nsi*I digested plasmid and the PCR fragment were co-transformed into SCMIG50 and colonies were selected on SDC-Trp. The overlapping sequences of both fragments allowed homologous recombination (figure 7.3) This yielded a functional plasmid, inserting the wild-type DNA into the digested plasmid (figure 7.3). Plasmids from colonies grown on SDC-Trp were recovered and p*CLA4* was identified by restriction analysis and sequencing.

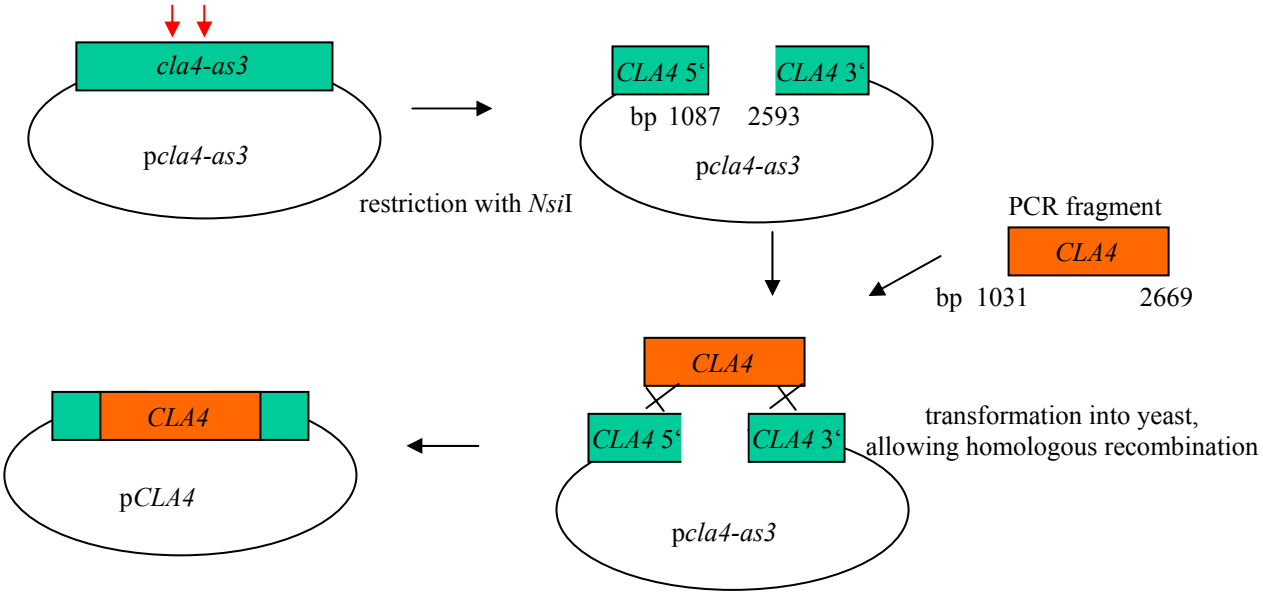

Figure 7.3: Construction of plasmid p*CLA4*

Plasmid p*cla4-as3* was cut with *Nsi*I, thereby removing the two as3 mutations (M659A, T701A; indicated with red arrows). A wild-type *CLA4* DNA fragment was amplified by PCR from chromosomal DNA, which has overlapping sequences to the cut plasmid. This fragment and the linearized plasmid were transformed into yeast, to allow homologous recombination and to generate p*CLA4*.

p*cla4-as3::URA3* was constructed by substituting *TRP1* from p*cla4-as3* by *URA3* by homologous recombination in yeast. For this purpose, a DNA fragment containing *URA3* and 40 nucleotides upstream of the ATG and downstream of the STOP of *TRP1* was amplified by PCR using the primers TRP1D.D and TRP1D.U and plasmid YDp-H as template. The DNA fragment was co-transformed with p*cla4-as3* into yeast and cells were selected on SDC-Ura, allowing replacement of the *TRP1* marker by *URA3*. Plasmids were recovered from yeast and p*cla4-as3::URA3* was identified by restriction analysis.

pGST-*myo5*-TEDS was obtained by PCR amplification of a *MYO5* DNA fragment encoding amino acids 322 to 391 using primers Myo5.966.BamHI.D and Myo5.1157.XhoI.U and pIN*MYO5* as template. The PCR fragment was digested with *BamH*I and *Xho*I and cloned into a *BamH*I/*Xho*I cut pGEX-5X-3 (Pharmacia), inserting the *MYO5* sequence in frame downstream of the glutathione-S-transferase.

To generate **pEG202*myo5*-H,** a DNA fragment encoding the head and neck domain of *MYO5* (aa 1 to 773) was amplified by PCR using primers MYO5.1D.EcoRI and XEIQ23U and p33*MYO5* as template. Subsequently, the PCR fragment was digested with *Eco*RI and *Xho*I and ligated into an *Eco*RI/*Xho*I cut pEG202 (kindly provided by Dr. R.Brent).

The **pJG4-5 series of plasmids containing different kinase genes** was made by amplification of the corresponding kinase-encoding genes with the listed primers and wild-type genomic DNA as template. The PCR fragments were digested with the listed restriction enzymes and cloned into pJG4-5 (kindly provided by Dr. R.Brent), which was cut with the same enzymes before, except for cloning using *Sma*I. In those cases, pJG4-5 was digested with *Eco*RI and the 5' overhangs were filled in by using the Klenow DNA polymerase fragment, yielding a blunt-ended DNA. Subsequently, the linearized plasmid was digested with *Xho*I and the *Sma*I/*Xho*I digested PCR fragments were ligated into it.

plasmid	primers	restriction enzyme
pJG4-5*PKH1*	PKH1.1D.EcoRI, PKH1.2301U.XhoI	*Eco*RI/*Xho*I
pJG4-5*PKH2*	PKH2.1D.EcoRI, PKH2.3243U.XhoI	*Eco*RI/*Xho*I
pJG4-5*YPK1*	YPK1.1D.SmaI, YPK1.2043U.XhoI	*Sma*I/*Xho*I
pJG4-5*YPK2*	YPK2.1D.EcoRI, YPK2.2034U.XhoI	*Eco*RI/*Xho*I
pJG4-5*ARK1*	ARK1.1D.SmaI, ARK1.1917U.XhoI	*Sma*I/*Xho*I
pJG4-5*PRK1*	PRK1.1D.EcoRI, PRK1.1914U.SalI	*Eco*RI/*Sal*I
pJG4-5*YCK1*	YCK1.1D.EcoRI, YCK1.1617U.XhoI	*Eco*RI/*Xho*I
pJG4-5*YCK2*	YCK2.1D.EcoRI, YCK2.1641U.XhoI	*Eco*RI/*Xho*I
pJG4-5*PKC1*	PKC1.1D.SmaI, PKC1.3456U.XhoI	*Sma*I/*Xho*I
pJG4-5*CLA4*	CLA4.1D.EcoRI, CLA4.2520U.SalI	*Eco*RI/*Sal*I
pJG4-5*IRE1*	IRE1.1698D.EcoRI*, IRE1.3345U.XhoI	*Eco*RI/*Xho*I
pJG4-5*CKA1*	CKA1.ID.EcoRI, CKA1.1119U.XhoI	*Eco*RI/*Xho*I
pJG4-5*CKA2*	CKA2.1D.EcoRI, CKA2.1020U.XhoI	*Eco*RI/*Xho*I
pJG4-5*CKB1*	CKB1.1D.EcoRI, CKB1.837U.XhoI	*Eco*RI/*Xho*I
pJG4-5*CKB2*	CKB2.1D.EcoRI, CKB2.777U.XhoI	*Eco*RI/*Xho*I

* without transmembrane domains

p33*ProtA-myo5-S357A* was generated by site-directed mutagenesis on p33*ProtA-MYO5* (constructed by Dr. F. Idrissi) using primers S357.DS and S357A. The mutation was confirmed by sequencing.

p33*ProtA-myo5-S357A*-H-*HA*$_3$ and p33*ProtA-MYO5*-H-*HA*$_3$ were constructed by homologous recombination in yeast, substituting the C-terminal part of *MYO5* or *myo5-S357A* after the head domain (aa 701-STOP) with 3 HA epitopes and the *HIS3* marker. For this purpose, a DNA fragment containing 40 nucleotides upstream of the Myo5p tail, followed by the 3 HA epitopes and a STOP codon, the *HIS3* marker and 40 nucleotides downstream of the *MYO5* STOP codon was amplified by PCR using primers MYO5.2061D.F2 and MYO5.3710U.R1 and pFA6A-3HA-HIS3MX6 as template. This DNA fragment was co-transformed with plasmids p33*ProtA-MYO5* or p33*ProtA-myo5-S357A* into yeast and cells were selected on SDC-Ura-His. The plasmids were recovered and p33*ProtA-MYO5*-H-*HA*$_3$ or p33*ProtA-myo5-S357A*-H-*HA*$_3$ were identified by restriction analyses.

pGST*MYO5-S1205A*-C and pGST*MYO5-S1205E*-C were constructed according to plasmid pGST*MYO5*-C (Geli *et al*., 2000). DNA fragments containing the TH2, SH3 and acidic domain (aa 984-STOP) of *myo5* mutants bearing either the S1205A or the S1205E mutation were amplified by PCR using primers M5.GST.2937.D and MYO5T and p33*myo5-S1205A* or p33*myo5-S1205E* as templates. These fragments were digested with *BamH*I and *Xho*I and ligated into the *BamH*I/*Xho*I digested plasmid pGEX-5X-3.

p33*myo5-S1205A* and p33*myo5-S1205E* were obtained by site-directed mutagenesis using p33*MYO5* as template and either primers Myo5.3593.S1205A.D and Myo5.3634.S1205A.U to generate the S1205A mutation, or primers Myo5.3593.S1205E.D and Myo5.3634.S1205E.U to generate the S1205E mutation. The mutations were confirmed by sequencing.

7.2.2 Primers

Table III. Primers

Name	Sequence*	Remarks*	Direction#
ko-skm1-F	CTTGCAGTGCAACATTGG		5’
ko-skm1-R	CTCCTAAATAAAAAAAAGCTAAATTTCAATGCCACTTT		
	AAGGATACTTTGCTATTTCTTAGCATTTTTGA		3’
PAKD1	TTTTCTCAATGGATCAGACG		5’
20LU1	TTTTTGATCTGTTGCCCG		3’
Cla4.1619.D	ATGCGGATCCATCTCAATGC		5’
Cla4.2529.U	TCATTCCTTCCACTCCAACAG		3’
Cla4.2509.YDp.D	CTGTTGGAGTGGAAGGAATGAGAATTCCCGGGGATCCG		

	G		5’
Cla4.2579.YDp.U	TACATAAGATTGTAGTATGTATGATATGCTTATAGAAAT		
	AGTTGTGTGCTAGCTAGCTTGGCTGCAGG		3’
Myo5.1.ApaI.D	GCCGGGGGCCCATGGCTATCTTAAAAAGAGGAGC	*Apa*I	5’
Myo5.3634.MluI.U	CAACCACGCGTTTACCAATCATCTTCCTCTTCATCTTC	*Mlu*I	3’
TRP1D.D	GTATACGTGATTAAGCACACAAAGGCAGCTTGGAGT		
	GAATTCCCGGGGATCCGGTGATG		5’
TRP1D.U	ACAAACAATACTTAAATAAATACTACTCAGTAATAAC		
	CTTGCAGGTCGACGGATCCGGTGATTG		3’
Cla4.1031.D	ATGTTCCCAACCAACAATATCC		5’
Cla4.2669.U	TTGATTATTGTATCGTTGCAGG		3’
Myo5.966.BamHI.D	GGAAGGAAGGATCCCAGTAACCGATTTTGTTGC	*Bam*HI	5’
Myo5.1157.XhoI.U	AAGGAAGGAACTCGAGTAACCCTACTCACAATCC	*Xho*I	3’
MYO5.1D.EcoRI	AACCAACCGAATTCATGGCTATCTTAAAAAGAGGAGCT		
	AG	*Eco*RI	5’
XEIQ23U	ACTGACTGGAATTCCTCGAGTCTTTCTTTTCTTCCACCCA	*Eco*RI/	
	AAAC	*Xho*I	3’
PKH1.1D.EcoRI	AACCAAGAATTCATGGGAAATAGGTCTTTGACAG	*Eco*RI	5’
PKH1.2301U.XhoI	AACCAACTCGAGTCATTTTTCATCTGTCCGTG	*Xho*I	3’
PKH2.1D.EcoRI	AACCAAGAATTCATGTATTTTGATAAGGATAATTCCATG	*Eco*RI	5’
PKH2.3243U.XhoI	AACCAACTCGAGTTACGACCTCTTCGATTTTGC	*Xho*I	3’
YPK1.1D.SmaI	AACCAACCCGGGAATGTATTCTTGGAAGTCAAAGTTTA		
	AG	*Sma*I	5’
YPK1.2043U.XhoI	AAGGAACTCGAGCTATCTAATGCTTCTACCTTGCACC	*Xho*I	3’
YPK2.1D.EcoRI	AACCAAGAATTCATGCATTCCTGGCGAATATC	*Eco*RI	5’
YPK2.2034U.XhoI	AACCAACTCGAGCTAACTAATGCTTCTCCCCTGC	*Xho*I	3’
ARK1.1D.SmaI	AACCAACCCGGGCATGAATCAACCTCAAATTGGC	*Sma*I	5’
ARK1.1917U.XhoI	AACCAACTCGAGTCACTTATCCAAGGATAACTTTCG	*Xho*I	3’
PRK1.1D.EcoRI	AACCAAGAATTCATGAATACTCCACAGATTAGTCTGTAT		
	G	*Eco*RI	5’
PRK1.1914U.SalI	AACCAAGTCGACTTAAACTTTGCTGGGAAACC	*Sal*I	3’
YCK1.1D.EcoRI	AACCAAGAATTCATGTCCATGCCCATAGCAAG	*Eco*RI	5’
YCK1.1617U.XhoI	AACCAACTCGAGTTAGCAACAACCTAATTTTTGGAA	*Xho*I	3’
YCK2.1D.EcoRI	AACCAAGAATTCATGTCTCAAGTGCAAAGTCCTT	*Eco*RI	5’
YCK2.1641U.XhoI	AACCAACTCGAGCTAACAGCATCCTAGCTTACTGAA	*Xho*I	3’
PKC1.1D.SmaI	AAGGAACCCGGGAATGAGTTTTTCACAATTGGAGC	*Sma*I	5’
PKC1.3456U.XhoI	AAGGAACTCGAGTCATAAATCCAAATCATCTGGC	*Xho*I	3’
CLA4.1D.EcoRI	AACCAAGAATTCATGTCTCTTTCAGCTGCAGC	*Eco*RI	5’
CLA4.2520U.SalI	AACCAAGCTGACTCATTCCTTCCACTCCAACAG	*Sal*I	3’

IRE1.1698D.EcoRI	AACCAAGAATTCGTATTATTATCCAAAATTGGATTTATG	*Eco*RI	5’
IRE1.3345U.XhoI	AACCAACTCGAGTTATGAATACAAAAATTCACGTAAAA		
	T	*Xho*I	3’
CKA1.ID.EcoRI	AACCAACCGAATTCATGAAATGCAGGGTATGGTCAG	*Eco*RI	5’
CKA1.1119U.XhoI	AACCAACCTCGAGTTATTTTTCAATTTGTTCCCTTATTG	*Xho*I	3’
CKA2.1D.EcoRI	AACCAACCGAATTCATGCCATTACCTCCGTCAAC	*Eco*RI	5’
CKA2.1020U.XhoI	AACCAACCCTCGAGTTATTCAAACTTCGTTTTGAAAAAC	*Xho*I	3’
CKB1.1D.EcoRI	AACCAACCGAATTCATGTCGCAAGAGTTTGTGGAAG	*Eco*RI	5’
CKB1.837U.XhoI	AACCAACCCTCGAGTTAAACCGCCGGTGTCTCG	*Xho*I	3’
CKB2.1D.EcoRI	AACCAACCGAATTCATGGGCAGTAGATCGGAGAATG	*Eco*RI	5’
CKB2.777U.XhoI	AACCAACCCTCGAGTTAGGTTTTAAAACCACCACTTTTC	*Xho*I	3’
S357.DS	GTATGAAAAGAGGG**GCA**GTGTATCATGTTC	S357A	5’
S357A	GAACATGATACAC**TGC**CCCTCTTTTCATAC	S357A	3’
MYO5. 2061D.F2	CCCACAGCAGGAGTACCAATTGGGTGTCACAAGTGTTTT		
	CCGGATCCCCGGGTTAATTAA		5’
MYO5.3710U.R1	TTTGCTCGTATAGAGTATATACTCGCTAAATACATTTTGA		
	GAATTCGAGCTCGTTTAAAC		3’
M5.GST.2937.D	ACACACACACGGATCCCCAGTTCCTCGCAAGCAAC	*BamH*I	5’
MY05T	AAGGAAGGAACTCGAGACCATGATTACGCCAAGCTTGC	*Xho*I	3’
Myo5.3593.S1205A.D	CCAATAAAATGAGATTAGAG**GCT**GATGACGAGGAGGCT		
	AACG	S1205A	5’
Myo5.3634.S1205A.U	CGTTAGCCTCCTCGTCATC**AGC**CTCTAATCTCATTTTATT		
	GG	S1205A	3’
Myo5.3593.S1205E.D	CCAATAAAATGAGATTAGAG**GAG**GATGACGAGGAGGC		
	TAACG	S1205E	5’
Myo5.3634.S1205E.U	CGTTAGCCTCCTCGTCATC**CTC**CTCTAATCTCATTTTATT		
	GG	S1205E	3’

* Either a restriction site (underlined) or an introduced mutation (bold) are given.
# Primer amplifying the coding strand are named 5’ primer, primer amplifying the complementary strand are 3’ primers.

7.3 Protein analyses

7.3.1 SDS-PAGE, immunoblots and antibodies

SDS-PAGE was performed as described (Laemmli, 1970) using a Minigel system (BioRad Laboratories, München). High and low range SDS-PAGE molecular weight standards (BioRad Laboratories, München) were used for determination of apparent molecular weights. Coomassie Brilliant blue staining was used for detection of total protein on acrylamide gels.

The protein concentration was determined with a BioRad Protein assay (BioRad Laboratories, München). To examine the expression of proteins, 15 µg or 30 µg (depending on the pocket size) of total protein were loaded on SDS-PAGE gels.

Immunoblots were performed as described (Geli *et al*., 1998). The Myo5p-specific tail antibody was described in Geli *et al*. (1998). The 3F10 and C4 monoclonal antibodies (Chemicon, Hofheim) were used for detection of the HA epitopes, and actin, respectively. Nitrocellulose membranes were stained with Ponceau Red for detection of total protein. Fluorescent and peroxidase-conjugated secondary antibodies were purchased from Sigma (Deisenhofen).

7.3.2 Quick yeast protein extract

Yeast cells were grown to an OD_{600} of about 0.4 (early exponential phase, around 10^7 cells/ ml). Approximately 20 x 10^7 cells were harvested, resuspended in 1 ml of sterile water and transferred to a 1.5 ml Eppendorf tube. Harvested cells were frozen and incubated at –20°C for at least 4 hrs. Subsequently, the pellet was dissolved in 30 µl of ice-cold IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml antipain) and glass bead lysed (5 x 1 min) on ice. Unbroken cells and debris were eliminated by centrifugation at 960 g for 5 min at 4°C.

7.3.3 LSP (low speed pelleted) yeast protein extract

These extracts were prepared according to Geli *et al*. (2000).

Yeast cells were grown to a density of approx. 4 x 10^7 cells/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) 50 mM sucrose. 1/10 pellet volume of XB 50 mM sucrose was added and the cells glass bead-lysed (10 x 1 min) on ice in the presence of protease inhibitors (0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml antipain). Unbroken cells and debris were eliminated by centrifugation at 2500 g at 4°C. The extracts were supplemented with sucrose (to 200 mM), the protein concentration was adjusted to 20 mg/ml, and finally, extracts were aliquoted, frozen in liquid N_2 and stored at –80°C until use.

Experiments in the presence of phosphatase inhibitors were performed with 10 mM sodium pyrophosphate, 10 mM NaN_3, 10 mM NaF, 0.4 mM EDTA, 0.4 mM $NaVO_3$, 0.4 mM Na_3VO_4, 2 µM cyclosporin A and 0.5 µM okadaic acid in LSP yeast extracts. Experiments in the presence of kinase inhibitors were performed with final 4 µM K252A and 1mM A3.

7.3.4 Purification of recombinant GST fusion proteins

GST-fusion proteins were purified from BL21 *E.coli* strains (Novagen, Bad Soden) according to Geli *et al*. (2000).

1/ 100 of an overnight *E.coli* culture was inoculated into minimal media (MM; Sambrook *et al*., 1989) containing 50 mg/l ampicillin. The culture was grown at 37°C to an OD_{600} of 0.4. Cells were shifted to 24°C and induced at an OD_{600} of 0.7-0.8 with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hrs. Cells were harvested and frozen at –20°C. For protein purification, a GST Amersham-Pharmacia (Freiburg) purification kit was used. Briefly, cells were thawed in PBS, 0.5 % Tween (for 1 l of cells 25 ml buffer) and lysed by sonication (10 x 30 sec). Cell debris was pelleted by centrifugation.

For the visual actin polymerization assay: 20 µl of 50 % glutathione Sepharose beads were added to the protein extracts obtained from 2 l of BL21 pGST*myo5-S1205A*-C, pGST*myo5-S1205E*-C or pGST*MYO5*-C cultures, and were incubated for 2 hrs shaking at 4°C. Beads were recovered by using econocolumns (BioRad Laboratories, München), washed several times and equilibrated in 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) 200 mM sucrose and finally adjusted to 50 %.

In vitro phosphorylations: 100 µl of 50 % glutathione sepharose beads were added to the protein extracts obtained from 2 l of BL21 pGST*MYO5*-C, pGST*myo5-S1205A*-C or pGST*myo5-S1205E*-C, respectively, 1.4 l of BL21-(SH3,TH2c), 1.2 l of BL21 pGST-(TH2c) or 1 l of BL21 pGST (pGEX-5X-3) cultures and incubated for 2 hrs shaking at 4°C. Beads were recovered by using econocolumns, washed several times and equilibrated in the appropriate buffers and finally adjusted to 50 %.

In vitro phosphorylation screen: 100 µl of 50 % glutathione Sepharose beads were added to the protein extracts of 2 l of BL21 pGST-*myo5*-TEDS or 1 l of BL21 pGST (pGEX-5X-3) cultures and incubated for 2 hrs shaking at 4°C. Beads were recovered by using econocolumns and washed several times.

7.4 The α-factor internalization assay

Saccharomyces cerevisiae cells exist in two haploid cells types, **a** or **α**, which can mate to form diploids (**a/α**). This conjugation pathway is initiated by binding of secreted mating a- and α-factor pheromones, secreted by *Mat***a** and *Mat***α** cells, respectively, to surface receptors of yeast cells of the opposite mating type. The α-factor receptor is Ste2p and the a-factor is Ste3p, which are exclusively expressed by *Mat**a*** and *Mat***α** cells, respectively. Binding of the ligands to the receptors lead to activation of signal transduction cascades that arrest cell division and

induce morphological changes, which are required for cell-cell fusion during mating. As part of the mechanism that allows pheromone desensitization and exit from cell cycle arrest after mating, mating factor binding induces internalization and degradation of the receptors (Chvatchko *et al*., 1986; Jenness and Spatrick, 1986; for review see Bardwell *et al*., 1994).

Receptor-mediated endocytosis in yeast can be measured by internalization of radiolabelled α-factor bound to its receptor, Ste2p (figure 7.4). This assay quantitatively measures the first step in endocytosis, the formation of the endocytic vesicle at the plasma membrane.

The principle of this assay is that α-factor can be released from uninternalized receptors, i.e. receptors at the cell surface, by washing with a low pH buffer (pH1). In contrast, washing with a pH6 buffer does not release the α-factor from its receptor. At the moment when the primary endocytic vesicle is pinched off from the cell surface, the α-factor can no longer be wash out from the cells by incubation with the pH1 buffer. The internalization kinetics of radiolabelled α-factor can be measured by monitoring the internalized cell-associated radioactive counts after pH1 buffer washing normalized versus the total (internalized plus surface bound) cell-associated counts after pH6 buffer washing per minute (figure 7.4; Dulic *et al*., 1991).

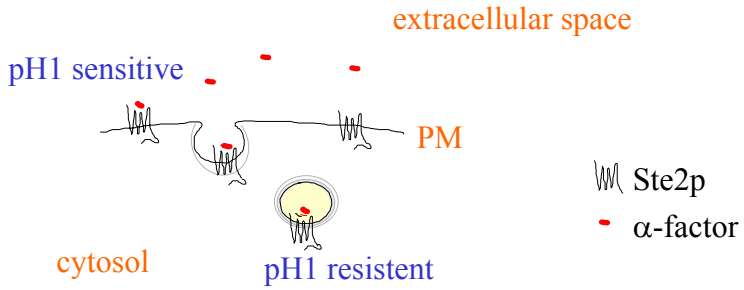

Figure 7.4: The assay for measuring α-factor internalization

The radiolabelled yeast pheromone α-factor binds to its receptor Ste2p at the cell surface and is subsequently internalized via endocytosis. The surface-bound but not the internalized α-factor can be removed by washing with a pH1 buffer. The ^{35}S-labelled α-factor internalization kinetics can be measured by monitoring the pH1 resistant counts (internalized) versus the pH6 resistant counts (total bound) per minute.

7.4.1 The uptake assay

[^{35}S] α-factor uptake assays were performed as described (Dulic *et al*., 1991).

For constitutive mutants, a pulse and chase protocol was used. Briefly, cells were grown to 0.5 - 1 x 10^7 cells/ml (early logarithmic phase), harvested and resuspended to 5 x 10^8 cells/ml in ice-cold YPD containing 100,000 dpm /ml of purified ^{35}S-α-factor and incubated for 45 min.

(For 6 time points, 60 x 10^7 cells were resuspended in 1.2 ml of ice-cold YPD.) Cells were pelleted again and the uptake was started by resuspension in 23°C YPD.

For temperature-sensitive (ts) and analogue (1NM-PP1; see below)-sensitive strains a continuous-presence protocol was used. Cells were grown to 0.5 - 1 x 10^7 cells/ml, harvested and resuspended to 5 x 10^8 cells/ml in prewarmed (23°C for the 1NM-PP1-sensitive strain and 37°C for temperature sensitive strains) YPD. In the case of the analogue-sensitive strain, 1NM-PP1 (4-amino-1-tert-butyl-3-(1-naphtylmethyl) pyrazolo [3,4-d] pyrimidine; kindly provided by E.Weiss and D.Drubin; Weiss *et al*., 2000) was added prior to the resuspension of the cells to the final concentrations of 25 µM or 100 µM (1.2 µl or 4.8 µl of a 25 mM 1NM-PP1 stock solution in DMSO, respectively), and cells were preincubated for 1 h before addition of 100,000 dpm/ml of purified ^{35}S-α-factor. For mock treatment, 1.2 µl or 4.8 µl DMSO were added. Temperature-sensitive strains were pre-incubated for 5, 15 or 30 min at restrictive temperature before addition of the α-factor.

2 x 100 µl of culture were taken at the indicated time points and the uptake was terminated by dilution (1/100) into ice-cold pH 1 (50 mM sodium citrate) or pH 6 (50 mM potassium phosphate) buffers, respectively. Cells were incubated for 20 min on ice to allow the dissociation of the α-factor from its receptor at pH1. Subsequently, cells were recovered by filtration onto GF/C filters (Whatman, Gerbershausen) and cell-associated counts were measured in a β-counter (Beckman LS 6000 TA). Internalized counts were calculated by dividing pH1-resistant (internalized) by pH6-resistant (total cell-bound) counts per time point.

The uptake rates correspond to the slope (internalized counts per minute) of the graphs at the linear, i.e. early, phase (typically within the first 10 min). The uptake rates of the wild-type strains in the individual experiments were arbitrarily set to 100 % and the uptake rates of the mutant strains were normalized to these 100 %. Uptake assays were performed at least three times and the mean and standard deviations calculated per time point. In all cases, the standard deviations were less than 10 % of the value.

7.5 Pulse and chase labelling of carboxypeptidase Y (CPY)

The experiment was done according to Stevens *et al*. (1982).

Yeast cells were grown at 23°C in SDYE media (0.67 % yeast nitrogen base, 0.2 % Bacto yeast extract, 2 % glucose, required amino acids) to approximately 10^7 cells/ml (early log phase). 13 x 10^7 cells (2.5 x 10^7 per time point + 0.5 x 10^7 cells) in 5.2 ml SD (0.67 % yeast nitrogen base, 2 % glucose, required amino acids) were pulsed for 5 min with 100 µCi of ^{35}S-labeling mix (^{35}S-methionine and ^{35}S-cysteine; Trans^{35}S-Label, ICN Biomedicals, Irvine,

USA), incubated for 5 min and chased by addition of cold 0.3 % methionine, 0.3 % cysteine, 300 mM $(NH_4)_2SO_4$. Samples were taken after 0, 5, 10, 15 and 30 min. For each time point, 1 ml of cells was added to 100 µl 10 x Stop solution (0.2M NaN_3, 0.2M NaF) on ice. Cells were subsequently pelleted and glass-bead lysed in 200 µl TEPI buffer (50 mM Tris pH 7.5, 5 mM EDTA, 5 µg/ml of each protease inhibitor chymostatin, leupeptine, antipain and pepstatin). SDS was added to 0.5 % and the samples were incubated at 95°C for 5 min, 800 µl TNET were added (30 mM Tris, pH 7,5, 120 mM NaCl, 5 mM EDTA, 1 % triton X-100), samples were mixed and cell debries were pelleted. 3 µl Antibody (α-CPY) and 35 µl (30% w/v) Protein A Sepharose (Sigma) were added to the supernatants and the immunoprecipitation was done for 2 hrs at RT. Pelleted immunocomplexes were washed with TNET + 0.1 % SDS, followed by TNET + 2 M urea and finally resuspended in 60 µl SDS-PAGE sample buffer. 20 µl were subjected to SDS gel electrophoresis and analyzed by autoradiography.

7.6 Fluorescence microscopy

7.6.1 F-actin staining with TRITC-phalloidin

TRITC-phalloidin staining was performed as described (Benedetti *et al*., 1994).

Cells were grown to approximately 1-2 x 10^7 cells/ml (early log. phase). 900 µl of this culture were prefixed for 30 min at room temperature (RT) by incubation with formaldehyde and Triton X-100 at final concentrations of 3.7 % and 0.04 %, respectively. Cells were then pelleted and resuspended in 1 ml of fixing buffer (35 mM KP_i, 4 % formaldehyde, 0.5 mM $MgCl_2$) and fixed for 2 hrs at RT. Subsequently, cells were washed with PBS and stained with 300 nM TRITC-phalloidin (Sigma) in PBS for 90 min at RT. Cells were washed with PBS, directly mounted on slides and visualized.

For experiments with the analogue-sensitive mutant, 3.6 µl of 25 mM 1NM-PP1 (final concentration: 100 µM; kindly provided by E.Weiss and D. Drubin (Weiss *et al*., 2000)) or 3.6 µl of DMSO (mock) were added previous to fixation.

7.6.2 Immunofluorescence

Immunofluorescence was performed based on Hicke *et al*. (1997).

Yeast cells were grown to 0.5-1 x 10^7 cells/ml (early log. phase). 20 ml of cells were fixed with a final concentration of 3.7 % formaldehyde in 100 mM KP_i, pH 6.5. After 2 hrs at RT, cells were harvested and spheroplasted with 52 U/ml of zymolyase in SP buffer (1.2 M sorbitol, 100 mM KP_i pH 6.5) for approximately 30 min at 30°C. Cells were attached to poly-L-lysine

coated slides and washed several times with PBT (PBS, 1 % BSA, 0.1 % Triton X-100). Cells were then incubated for 1 h with a 1:50 dilution of the primary antibody (rat α-HA, Chemicon, Hofheim) in PBT. Subsequently, cells were washed with PBT and incubated for 30 min with a 1:100 dilution of the CY3-conjugated secondary antibody (α-rat, Dianova, Hamburg) in PBT. Cells were washed with PBT and PBS, mounted in Mowiol (Calbiochem, San Diego, USA) and visualized using a fluorescence microscope.

7.6.3 Visual actin polymerization assay

The actin polymerization assay was performed according to Geli *et al*. (2000) and Idrissi *et al*. (2002).

Briefly, 7 µl of LSP yeast extracts (see above) were mixed with 1 µl of ARS (ATP regenerating system; 10 mg/ml creatine kinase, 10 mM ATP, 10 mM $MgCl_2$, 400 mM creatine phosphate) and 1 µl of 10 µM rhodamine-actin (APHR-C, Cytoskeleton, Inc). The polymerization reaction was initiated by addition of 1 µl 50 % of either GST-Myo5-Cp-Sepharose, GST-Myo5-S1205A-Cp-Sepharose or GST-Myo5-S1205E-Cp-Sepharose beads.

Samples were incubated at RT (26°C) unless otherwise mentioned. Samples were visualized after 15 – 30 min using a fluorescence microscope.

7.7 Phosphorylation experiments

7.7.1 *In vivo* phosphorylation assay

7.7.1.1 Media and buffers

SDC low phosphate:

2 % glucose, 1 g/l KCl, 0.1 g/l NaCl, 1 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgCl_2$ x 6 H_2O, 0.1 g/l $CaCl_2$ x 2 H_2O, 10 nM H_3BO_3, 10 nM $CuCl_2$, 100 nM KI, 50nM $FeCl_3$, 70 nM $ZnCl_2$, 2 µg/l biotin, 0.4 mg/l calcium pantothenate, 2 mg/l inositol, 0.4 mg/l niacin, 0.2 mg/l p-aminobenzoic acid, 0.4 mg/l pyridoxin, 0.4 mg/l thiamin, 0.2 mg/l riboflavin, 50 mg/l tryptophane, 20 mg/l adenine, 20 mg/l histidine, 50 mg/l leucin, 50 µM KH_2PO_4;

when growing wild-type strains, the media was supplemented with 20 mg/l uracil.

SDC no phosphate:

2 % glucose, 1 g/l KCl, 0.1 g/l NaCl, 1 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgCl_2$ x 6 H_2O, 0.1 g/l $CaCl_2$ x 2 H_2O, 10 nM H_3BO_3, 10 nM $CuCl_2$, 100 nM KI, 50nM $FeCl_3$, 70 nM $ZnCl_2$, 2 µg/l biotin, 0.4 mg/l calcium pantothenate, 2 mg/l inositol, 0.4 mg/l niacin, 0.2 mg/l p-aminobenzoic acid, 0.4

mg/l pyridoxin, 0.4 mg/l thiamin, 0.2 mg/l riboflavin, 50 mg/l tryptophane, 20 mg/l adenine, 20 mg/l histidine, 50 mg/l leucin;
when growing wild-type strains, the media was supplemented with 20 mg/l of uracil.

IP buffer

50 mM Tris-Cl, pH 7.5
150 mM NaCl
5 mM EDTA
phosphatase inhibitors:
10 mM sodium pyrophosphate
10 mM NaN_3
10 mM NaF
0.4 mM EDTA
0.4 mM $NaVO_3$
0.4 mM Na_3VO_4
2 μM cyclosporin A
0.5 μM okadaic acid

7.7.1.2 Phosphorylation assay

Yeast cells were grown to 0.5-1 x 10^7 cells/ml (early log. phase). 10^8 cells were harvested (2400 g, 5 min) and washed once with water. The pellet was resuspended in 20 ml of SDC low phosphate media and incubated at 23°C for 5 hrs. Cells were then repelleted (2400 g, 5 min), washed once with SDC no phosphate media, transferred to a 2 ml Eppendorf tube and finally resuspended in 1 ml of SDC no phosphate. 1 mCi of radioactive PO_4^{3-} (ICN Biomedicals, Irvine, USA) was added and cells were allowed to internalize the radioactive phosphate for 30 min at RT (26°C). Subsequently, cells were harvested and washed twice with IP buffer. The pellet was then frozen at –20°C.

After thawing, cells were broken in 100 μl of IP buffer containing protease inhibitors (0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml antipain), phosphatase inhibitors and approximately 200 mg of glass beads by vortexing 10 times for 1 min, with 1 min incubation on ice intervals. Triton-X 100 was added to 1 %, cell debris was pelleted and the supernatant transferred to a new tube. 20 μl of a Myo5p-specific antibody (Geli *et al*., 1998) pre-bound to 40 μl of 50% protein A sepharose (Amersham-Pharmacia, Freiburg) were added and the immunoprecipitation was performed for 2 hrs on ice. In case of Protein A fusion

proteins, 40 µl of 50 % IgG Sepharose (Amersham-Pharmacia, Freiburg) were used for the immunoprecipitations.

The beads were pelleted and washed several times with IP buffer with and subsequently without Triton-X 100. The beads were finally boiled in 20 µl SDS-PAGE buffer and half of the eluate was loaded on either a 7.5 % or a 10 % SDS-PAGE gel.

7.7.2 *In vitro* phosphorylation assays

7.7.2.1 *In vitro* phosphorylation with protein extracts

Required GST fusion proteins were purified as described in section 7.3.4. For each phosphorylation reaction, 10 µl of GST-fusion protein containing beads were mixed with 10 µl empty glutathione beads (to increase the amount of visible beads). 28 µl LSP protein extract and 20 µCi γ-^{32}P-ATP (Amersham-Pharmacia, Freiburg) were added and the reaction was allowed to proceed for 30 min at 23°C. The beads were washed 3 times with PBS containing protease inhibitors (0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml antipain) and finally boiled in 30 µl SDS-PAGE sample buffer. 15 µl were loaded on a 15 % SDS-PAGE gel.

7.7.2.2 *In vitro* phosphorylation by casein kinase II (CKII)

GST fusion proteins were purified as described in section 7.3.4. The phosphorylation was performed according to the manufacturers instructions (Calbiochem, San Diego, USA). Briefly, for one phosphorylation reaction, 10 µl of GST-fusion protein coated beads were mixed with 10 µl empty glutathione beads (to increase the amount of visible beads). 7.5 µl phosphorylation buffer (20 mM Tris pH 7.5, 50 mM KCl, 10 mM $MgCl_2$), 1.98 µl of 1 mM ATP, 0.1 µl of recombinant human CKII (Calbiochem, San Diego, USA) and 0.4 µCi [γ-^{32}P]-ATP (Amersham-Pharmacia, Freiburg) (0.4 µl) were added and incubated for 30 min at 30°C. The beads were pelleted, washed 3 times with PBS, and subsequently boiled in 30 µl SDS-PAGE sample buffer. Half of the eluate was loaded on a 15 % SDS-PAGE gel.

7.8 *In vitro* phosphorylation screen (chip assay)

For the *in vitro* phosphorylation screen the GST and GST-myo5-TEDSp proteins were purified as described in section 7.3.4 and subsequently eluted with 20 mM glutathione, 100 mM HEPES pH7.7, 1 % Triton-X100 (100 µl of 50 % beads were eluted with 300 µl buffer) and dialysed over night against 20 mM HEPES pH 7.7. Proteins were lyophilized for sending to Dr. H. Zhu by using an Alpha1-2 lyophilisator (Christ, Osterode). The *in vitro* phosphorylation

screen was performed as described in Zhu *et al*. (2000). Briefly, substrates were crosslinked to a silicone elastomer, polydimethylsiloxilane (PDMS) multiple well (1.4 mm in diameter) plates, using 3-glicidoxyprolyltrimethoxysilane (GPTS) as crosslinker. Samples were blocked with 1 % BSA and subsequently incubated for 30 min at 30°C in the presence of [γ-^{33}P]-ATP and 119 different GST-kinase fusion proteins purified from yeast (3 out of the 122 kinases identified in the yeast genome could not be purified using the standard protocol). After extensive washing, phosphorylation signals were quantified by phosphoimager and normalized against the media for each kinase.

7.9 Two-hybrid techniques

The Interaction Trap two-hybrid system was used (Gyuris *et al*., 1993). Plasmids pEG202, pJG4-5, pRFHM-1 and pSH18-34 and the strain EGY48 were obtained from Dr. R. Brent (MGM, Boston). To measure β-galactosidase activity, EGY48 bearing the lexAop-lacZ reporter plasmid pSH18-34, was co-transformed with the appropriate pEG202 and pJG4-5 derived plasmids and streaked out on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing plates (0.67 % yeast nitrogen base (Difco, Heidelberg), 7 g/l Na_2HPO_4, 3 g/l NaH_2PO_4, 2 % galactose, 1 % raffinose, 40 mg/ml leucine, 80 mg/l X-Gal (Sigma, Deisenhofen), 2 % agar, pH 7). Pictures were taken after 2-3 days of growth at 30°C.

7.10 Plasmid recovery from yeast

5 ml of the yeast culture were grown to stationary phase in the appropriate minimal media. Cells were pelleted and resuspended in 0.4 ml of lysis buffer (0.5 M NaCl, 0.2 M Tris pH 7.5, 10 mM EDTA, 1 % SDS). 150 µl of glass beads and 300 µl of phenol:chloroform:isoamyl alcohol were added and the mixture was vortexed for 2 min. After centrifugation, the aqueous phase was recovered and mixed with phenol:chloroform:isoamyl alcohol and separated again by centrifugation. Finally, the plasmid-DNA was precipitated from the aqueous phase with 2.5 volumes of ethanol. The DNA was resuspended in 50 µl of water and 3 µl were electroporated into *E.coli*.

7.11 Miscellaneous

Chemicals were obtained from Roth (Karlsruhe), Sigma (Deisenhofen), Fluka (Steinheim), Merck (Darmstadt) or Molecular Probes (Leiden, Netherlands). Radiochemicals were from ICN Biomedicals (Irvine, USA) and Amersham-Pharmacia (Freiburg or Little Chalfont,

England). Enzymes for molecular biology were obtained from New England Biolabs (Frankfurt a.M.) or Roche (Mannheim).

Culturing of *E. coli* and *S. cerevisiae* were performed as described (Guthrie and Fink, 1991; Sambrook *et al*., 1989; Sambrook and Russel, 2001).

Standard DNA manipulations (gel electrophoresis, enzymatic digestion, ligation, transformation, plasmid preparation and polymerase chain reaction) were performed as described (Sambrook *et al*., 1989; Sambrook and Russel, 2001).

Plasmids were purified with a Nucleobond plasmid purification kit from Macherey-Nagel (Dueren; Midi- or Maxiprep-set). DNA in agarose gels was purified using the gel extraction kit from Qiagen (Hilden).

Electroporation of *E. coli* cells was performed as described (Dower *et al*., 1988).

PCRs were performed using a DNA polymerase with proof-reading activity (Vent polymerase or Pfu polymerase; New England Biolabs, Frankfurt a.M.) on a TRIO-thermoblock (Biometra GmbH, Göttingen). Oligonucleotides were synthesized by Interactiva/Thermo Hybaid (Freiburg).

DNA sequencing was performed by TopLab (München). Site directed mutagenesis was performed using the QuickChange kit from Stratagene (LaJolla, USA).

For fluorescence and phase contrast microscopy an Axiovert 35 fluorescence microscope (Carl Zeiss, Jena) was used.

8 Publications

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