PHOSPHATIDYLINOSITOL (4,5)-BISPHOSPHATE TURNOVER BY INP51 REGULATES THE CELL WALL INTEGRITY PATHWAY IN *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Signal transduction pathways are important for the cell to transduce external or internal stimuli where second messengers play an important role as mediators of the stimuli. One important group of second messengers are the phosphoinositide family present in organisms ranging from yeast to mammals. The dephosphorylation and phosphorylation cycle of the phosphatidylinositol species are thought to be important in signaling for recruitment or activation of proteins involved in vesicular transport and/or to control the organization of the actin cytoskeleton. In mammals, phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) signaling is essential and regulated by various kinases and phosphatases. In the model organism *Saccharomyces cerevisiae* $PI(4,5)P_2$ signaling is also essential but the regulation remains unclear. My dissertation focuses on the regulation of $PI(4,5)P_2$ signaling in *Saccharomyces cerevisiae*.

The organization of the actin cytoskeleton in *Saccharomyces cerevisiae* is regulated by different proteins such as calmodulin, CMD1, and here I present data that CMD1 plays a role in the regulation of the only phosphatidylinositol 4-phosphate 5-kinase, MSS4, in *Saccharomyces cerevisiae.* CMD1 regulates MSS4 activity through an unknown mechanism and thereby controls the organization of the actin cytoskeleton. MSS4 and CMD1 do not physically interact but MSS4 seems to be part of a large molecular weight complex as shown by gel filtration chromatography. This complex could contain regulators of the MSS4 activity. The complex is not caused by dimerization of MSS4 since MSS4 does not interact with itself.

Two pathways, the cell wall integrity pathway and TORC2 (target of rapamycin complex 2) signaling cascade are important for the organization of the actin cytoskeleton. Loss of TOR2 function results in a growth defect that can be suppressed by MSS4 overexpression. To further characterize the link between MSS4 and the TORC2 signaling pathway and the cell wall integrity pathway we looked for targets of $PI(4,5)P_2$. The TORC2 pathway and the cell wall integrity pathway signal to the GEF ROM2, an activator of the small GTPase RHO1. In our study we identified ROM2 as a target of $PI(4,5)P_2$ signaling. We observed that the ROM2 localization changes in an *mss4* conditional mutant. This suggests that the proper localization needs $PI(4,5)P_2$. This could be mediated by the putative $PI(4,5)P_2$ binding pleckstrin homology (PH) domain of ROM2.

To better understand the regulation of PI(4,5)P_2 levels in *Saccharomyces cerevisiae* we focused on one of the PI(4,5)P_2 5-phosphatases, INP51. Here we present evidence that INP51 is a new negative regulator of the cell wall integrity pathway as well as the TORC2 pathway. INP51 probably regulates these two pathways by the turnover of PI(4,5)P_2 thereby inactivating the effector/s. The deletion of *INP51* does not result in any phenotype, but when combined with mutations of the cell wall integrity pathway we observe synthetic interaction.

INP51 together with the GTPase activating protein (GAP) SAC7, responsible for the negative regulation of RHO1, negatively regulates the cell wall integrity pathway during vegetative growth. One of the targets of cell wall integrity pathway, the cell wall component chitin, which is normally deposited at the bud end, bud neck and forms bud scars, is delocalized in the mother cell in the *sac7 inp51* double deletion mutant. In addition, another downstream component of the cell wall integrity pathway, the MAP kinase MPK1, has increased phosphorylation and protein level in the *sac7 inp51* double deletion mutant. This suggests that INP51 is important for the negative regulation of the cell wall integrity pathway.

Furthermore, we show evidence that INP51 forms a complex with TAX4 or IRS4, with two EH-domain containing proteins, that positively regulates the activity of INP51 and in this manner negatively regulate the cell wall integrity pathway. The EH-domain is known to bind the NPF-motif. This motif is present in INP51 and is important for INP51 interaction with TAX4 or IRS4. The EH-NPF interaction is a conserved mechanism to build up protein networks. The interaction between an EH-domain containing protein and a PI(4,5)P_2 5-phosphatase is conserved. This is demonstrated by the epidermal growth factor substrate EPS15 (EH) interaction with the PI(4,5)P_2 5-phosphatase synaptojanin the mammalian orthologue of the *Saccharomyces cerevisiae* INP proteins.

In summary, INP51 together with TAX4 and IRS4, forms complexes important for regulation of PI(4,5)P_2 levels. The complexes are linked to the TORC2 signaling pathway and the cell wall integrity pathway, specifically regulating MPK1 activation and chitin biosynthesis. The work presented in this dissertation facilitates the development of a model of the complex regulation of PI(4,5)P_2 signaling in *Saccharomyces cerevisiae*.

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TABLE OF CONTENTS

1. INTRODUCTION

1.1 Phosphoinositides and phosphoinositide-binding modules

Cells transduce external or internal stimuli through different signaling mechanisms. Stimuli can be transduced by protein modification such as phosphorylation, prenylation or palmitoylation and by second messengers such as phosphoinositides to change the activity status of a protein. Protein modifications take place on conserved motifs within the target protein and are often carried out by specific enzymes. One well-studied example of protein modification is the prenylation of the RAS protein in mammalian cells. The RAS protein contains a specific sequence that is recognized by an enzyme called farnesyl transferase protein. Once RAS is prenylated, it can attach to the plasma membrane and function in signal transduction (1). In addition, second messengers that bind the target protein through specific domains can mediate signal transduction. Calcium (Ca^{2+}) is a second messenger that increases in the cytosol upon stimuli such as hormonal activation and binds to specific domains like the C2 domain (2,3). Another important group of second messengers acting analogous to Ca^{2+}, is the lipid family which are involved in different cellular processes such as regulation of cell growth and of the organization of the actin cytoskeleton (4-6).

1.1.1 Phospholipids involved in regulatory processes

Lipids and their metabolites are known primarily to have an essential structural role involving the building up of membranes that build up a barrier between the cytoplasm and the external environment. In addition, these molecules are responsible for compart-mentalization of a cell (7). Lipids also have an essential role in regulatory processes such as phosphoinositide signaling the focus of this introduction (8). Lipids are a diverse group of biological molecules some made up primarily of and others exclusively of nonpolar groups. Lipids can be classified into three major groups: neutral lipids, phospholipids and steroids. Neutral lipids are the major components of fats and oils used for storage {Alberts, 2002 #341}. The primary structural element of biological membranes is the phospholipid group that comprises phosphate-containing molecules with structures related to the

triglycerides and consist of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (PI) (Figure 1){Alberts, 2002 #341}.

PI is the building block of a subgroup of the phospholipid family, the phosphoinositides (PPI) (Figure 2) that have become important in the past two decades since they have specific roles as second messengers (8). PPI are produced in different compartments of the cell and play an important role in recruiting proteins via specific domains (9). PPI are unique among second messengers because they can be rapidly modified by phosphorylation or dephosphorylation to induce or inhibit signaling (10).

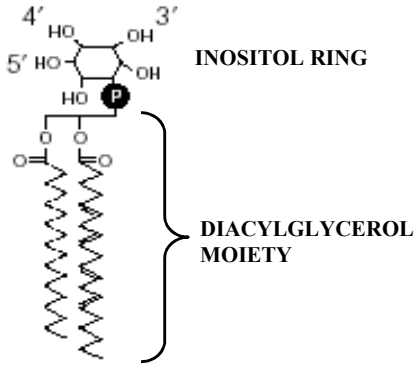

Figure 1 **Chemical structure of phosphatidylinositol**.

Phosphatidylinositol is built up of an inositol ring linked to a diacelglycerol moiety that contains two fatty acid chains. There are free hydroxyl group at positions 2-6 on the inositol head group. Only phosphoinositides phosphorylated at the 3', 4' and/or 5' have been identified in mammalian cells (11).

1.1.2 Synthesis of phosphatidylinositol and its derivatives, the phosphoinositides

PI is the starting molecule for synthesis of PPI (Figure 1) that consists of an inositol ring (cyclic hexadydric alcohol) and the hydrophobic component diacylglycerol (DAG) moiety Figure 2). The DAG moiety contains two fatty acid chains esterified onto glycerol which is in turn attached through a diester phosphate to the 1-hydroxyl of the inositol ring (12). PPI is produced by phosphorylation thereby producing different PI species (13).

PI is essential because *Saccharomyces cerevisiae* cells with mutations in the *PIS1* gene encoding the phosphatidyltransferase responsible for generating PI (Figure 2) are not viable

(13,14). The lethality is probably not due to an effect on membrane structure but rather due to its role in cellular signaling such as in the organization of the actin cytoskeleton and membrane trafficking (8).

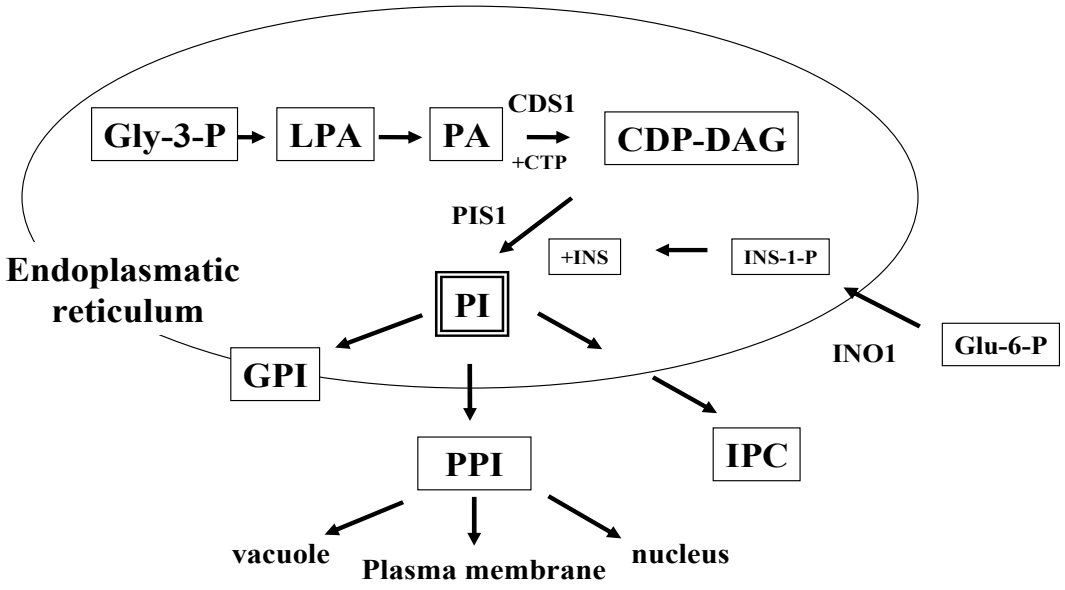

Figure 2 **The biosynthesis of phosphatidylinositol**

Gly-3-P is used to generate LPA and further PA. CTP is introduced into PA by CDS1 (localized in the ER) to yield CDP-DAG that in turn is the substrate of PIS1 (localized in the ER) that generates phosphatidylinositol (PI). PI can be further processed in three branches. PI is used in the biosynthesis of GPI initiated by the transfer of *N*-acetylglucosamine to phosphatidylinositol. The AUR1 protein catalyzes the reaction in which inositol phosphate is transfered from phosphatidylinositol to IPC in the Golgi. The third branch is the phosphatidylinositols phosphorylated at different locations of the cell. Glycerol-3-phosphate (Gly-3-P); lysophosphatidic acid (LPA); phosphatidic acid (PA); cytidine diphosphate-diacylglycerol (CDP-DAG); phosphatidylinositol (PI); glycosyl phosphatidylinositol (GPI); phosphorylated phosphatidylinositol (PPI) and inositol phosphate ceramide (IPC). *Modified from (8)*

For the synthesis of PI, glycerol-3-phosphate (Gly-3-P) undergoes two steps of processing to yield phosphatidic acid (PA). PA is then used as the substrate of cytidine 5-bisphosphate-diacylglycerol (CDP-DAG) synthase (CDS1) to produce CDP-DAG (13,15).

This is followed by the transfer of CDP-DAG to the inositol ring to produce PI that is modified further by phosphorylation (Figure 1 and 2). In addition, PI is also used for glycosylphoshpatidylinositol and sphingolipid synthesis (14).

The inositol ring can be phosphorylated and dephosphorylated by different but specific kinases and phosphatases yielding a wide combination of signaling molecules. These species (PPI) are the result of differential phosphorylation on the 3', 4' and 5' position of the inositol ring (Figure 1). There are five phosphoinositide species in *Saccharomyces cerevisiae*: phosphatidylinositol (3)-phosphate (PI(3)P), phosphatidylinositol (4)-phosphate (PI(4)P), phosphatidylinositol (3,5)-bisphosphate ($PI(3,5)P_2$) and phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$). In mammalian cells two additional species, phosphatidylinositol (5)-phosphate (PI(5)P) and phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$), exist (16).

The first example of phosphoinositide signaling was the generation of the second messengers inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) by hydrolysis of $PI(4,5)P_2$ (6). It was later established that $PI(4,5)P_2$, the main focus in this introduction, and other PPI are used directly for regulatory functions (17). PPI mediate their function by forming membrane-binding sites for soluble proteins that contain specific PPI-binding modules.

1.1.3 Phosphoinositide signaling mediated by phosphoinositide-binding domains

The phosphoinositide-binding domains such as the pleckstrin homology (PH) domain are conserved from yeasts to mammals. However, there are few cases where PPI binds proteins independently of known phosphoinositide-binding domains such as actin-associated proteins that bind $PI(4,5)P_2$ through electrostatic interaction using clusters of positively charged residues (18,19).

The identification of lipid binding domains has made it possible to elucidate the function of the different PPI and the cellular processes where PPI is involved. The specificity of each phosphoinositide is based also on its structure and localization (9). PPI are involved in recruiting proteins, stabilizing protein complexes or activating membrane proteins and their synthesis takes place at different cellular locations such as the plasma membrane and the

nucleus (6,9,10,16,20). The same domains that bind PPI in cytosolic proteins are also present in nuclear proteins where equivalent signaling via PI(4,5)P_2 takes place (21). PPI have been implicated in a diverse range of functions, from signal transduction (e.g. insulin signaling), regulation of GTPases and their cofactors in vesicle budding, endosome fusion, cytoskeletal reorganization to differentiation, cell survival and platelet activation (9,10,22,23).

The different phosphoinositide-binding domains generally bind a specific phosphoinositide and are distinct in structure, such as the PX-domain that only binds PI(3)P, but comparison studies show that they do share common features such as a positively charged binding core. The PPI interaction is generally weak allowing a flexible association to the protein. It is believed that this mechanism could allow a local enrichment or translocation of the specific target protein upon increased phophoinositide production (24).

1.1.3.1 PI(4,5)P_2-binding modules.

The first domain shown to bind a phosphoinositide, PI(4,5)P_2 in this case, was the 100 amino acid long pleckstrin homology (PH) domain found in pleckstrin the major substrate of protein kinase C in platelets (25). The PH domain is mainly found in proteins involved in the regulation of the actin cytoskeleton. The group of PH domains (approximately 250 in the human proteome and 25 in *Saccharomyces cerevisiae*) are not homologous and are found to have various specificities (10). PH domains have not only been shown to bind PPI but also inositol polyphosphates. Furthermore in some cases the PH domains have been shown to be involved in protein-protein interactions (26). In vitro experiments show that the PH-containing proteins can be divided into different groups depending on their phosphoinositide specificity. PH domains can bind PI(3,4,5)P_3, PI(4,5)P_2 and PI(3,4)P_2. Interestingly, only a minority of the existing PH domains bind a particular PPI with high affinity. One reason could be that PI(4,5)P_2 is more abundant and does therefore not require stringent affinity (10). However, in general the function of the PH domain is phosphoinositide-dependent. Even if the PH domains are a heterogenous group on the amino acid sequence level but interestingly the three-dimensional structures of known PH domains are highly conserved (18).

PH domains are found in signaling molecules such as protein kinases, phospholipases and positive regulators of small GTPases such as GEF involved in the organization of the actin cytoskeleton (27). Interestingly, not only guanine nucleotide exchange factors, positive regulators of small GTPases, are regulated by binding to PPI but also the GTPase activating proteins the negative regulators of small GTPases (22,28,29).

The ENTH (epsin N-terminal homology) domain is an additional $PI(4,5)P_2$ binding domain and is mainly found in proteins involved in endocytosis (30). In mammalian cells, the HIPR1 (Huntingtin interacting-protein related protein 1) contains an ENTH and an actin-binding module. It localizes to clathrin-coated vesicles suggesting a link between endocytosis and the actin cytoskeleton (24). In contrast, the ENT3 protein in *Saccharomyces cerevisiae* involved in protein sorting binds $PI(3,5)P_2$ through its ENTH domain adding more complexity to suggested specific $PI(4,5)P_2$-binding proteins (31). Another newly identified $PI(4,5)P_2$-binding domain is the postsynaptic density (PDZ) domain implicated in regulating the assembly of multiprotein complexes at the cell membrane (30).

Furthermore, $PI(4,5)P_2$ has been shown to modulate the activity of ion channels in mammalian cells but the binding domain has not been described (32). $PI(4,5)P_2$ has been suggested to either bind directly to the channel to modulate activity or alter local membrane topology by electrostatic interactions (11). An additional PH domain independent signalling is exemplified by Arp2/3 complex involved in promoting actin polymerization that is regulated by the WASP family (18,19). $PI(4,5)P_2$ stimulates the Wiskott-Aldrich syndrome protein (WASP) synergistically with CDC24 signaling and SH3-containing adapter proteins (19). Interestingly, WASP has a PH-like domain but it is a lysine-rich basic region that most likely mediates the phosphoinositide binding (33).

1.1.3.2 The FYVE domain and PX domain

Membrane targeting can also be mediated by PI(3)P and $PI(3,5)P_2$ through the FYVE and the PX domains.

The FAB1/YOTB/VAC1/EEA1 (FYVE) domain is important for the regulation of vesicle-mediated protein transport and binds PI(3)P with high specificity and has not been observed to bind other PPI (26,34). The high binding affinity of PI(3)P is achieved by

head-group interaction but requires a stabilization through membrane association and/or oligomerization (10). The human early endosomal antigen **(**EEA1) concentrates at early endosomes in part through specific binding of its FYVE domain to PI(3)P (35,36). In *Saccharomyces cerevisiae* the FYVE domain is present in proteins involved in vesicular trafficking. One example is the FAB1 protein, a PI (3)-P 5-kinase that contains a central FYVE domain that might recruit FAB1 to the endosomal fraction rich in PI(3)P substrate. Furthermore, the FYVE domain has been linked to the actin cytoskeleton which is exemplified by the frabin protein also involved in endosomal trafficking (37).

The phlox (phagocyte oxidase) homology (PX) domain also known as PB2 (phlox and Bem1p 2) domain, is associated with signaling and membrane trafficking (27). The PX domain plays in most cases a membrane targeting role binding PI(3)P in a similar way as the FYVE domain observed in $p40^{phox}$ subunit of the phagocyte NADPH oxidase. The NADPH oxidase responds to PI 3-kinase signaling in neutrophil function and inflammation by producing reactive oxygen species (ROS) (38). In the $p47^{phox}$ subunit of the NADPH oxidase the PX domain binds $PI(3,4)P_2$ (27,38). The PX domain has also been observed as a protein-protein interaction module. The same PX domain in $p47^{phox}$ is able to interact with the SH3 domain from the same protein (39). The dual interaction to SH3 and PPI might reflect a mode of regulation. The PX domain also occurs in proteins involved in membrane trafficking, such as sorting nexus and in the yeast vacuole protein VAM7 (40,41). The polarity establishment proteins BEM1 and BEM3 in budding yeast contain the PX domain regulating the localization (42).

In addition to domains specifically binding to PI(3)P there is evidence for new motifs for lipid-protein interaction. The yeast protein ETF1 binds PI(3)P but does not contain a previously characterized PPI-binding motif but contains a $K_{113}KPAKK_{118}$ sequence that might be important for phosphoinositide binding. ETF1 has been suggested to be a specific VPS34 effector involved in autophagy (43).

1.2 Phosphoinositide synthesis, turnover and function in higher eukaryots

PPI binding to different proteins is controlled by specific kinases and phosphatases that regulate the PPI levels. The synthesis and turnover of PPI are well characterized in mammalian cells where most enzymes exists as a variety of isoforms with diverse functions in different cell types (6). Their essential role has been proven by the fact that mutations in enzymes involved in phosphoinositide signaling have been implicated in human diseases (34). Therefore, the regulation of PPI signaling is very important. PPI levels can be regulated by phosphatases that specifically remove the 3', 4' and 5' phosphates on the inositol ring (14). Furthermore, PI(4,5)P_2 signaling can be inhibited by hydrolysis mediated by phospholipase C.

1.2.1 PI(3,4,5)P_3 and the insulin signaling pathway

In mammals, three classes of phosphatidylinositol 3-kinase (PI 3-K) enzymes exist (class I, II and III) (Figure 3). They are mainly involved in growth regulation but also in other cellular processes (11). The class I enzymes use PI, PI(4)P or PI(4,5)P_2 as substrate *in vitro*. *In vivo* they mainly produce PI(3,4)P_2 and PI(3,4,5)P_3 where PI(3,4)P_2 could be the result of the action of a 5-phosphatase on PI(3,4,5)P_3. Class II enzymes phosphorylate PI and PI(4)P. Class III can only phosphorylate PI and it is thought to be involved in membrane trafficking (11,44).

In the insulin signaling pathway (Figure 4), the Class I PI 3-kinase responds to insulin by converting the plasma membrane lipid PI(4,5)P_2 to PI(3,4,5)P_3 (45). PI(3,4,5)P_3 then binds to the PH domain of one of the major targets of PI 3-kinase signaling, the Akt protein (also known as protein kinase B (PKB)). The importance of the Akt PH domain for activation depends on the cell type and/or the stimulus. Interestingly, activation of the PI 3-K/Akt pathway has been implicated in the prevention of apoptosis in several cell types (46). Additional studies, has implicated PI 3-K deregulation in various cancers (47). Regulation of the PI 3-K signaling is therefore important to avoid improper signaling. Turnover and regulation of PI 3-K products is mediated by two phosphatases PTEN and SHIP1/2.

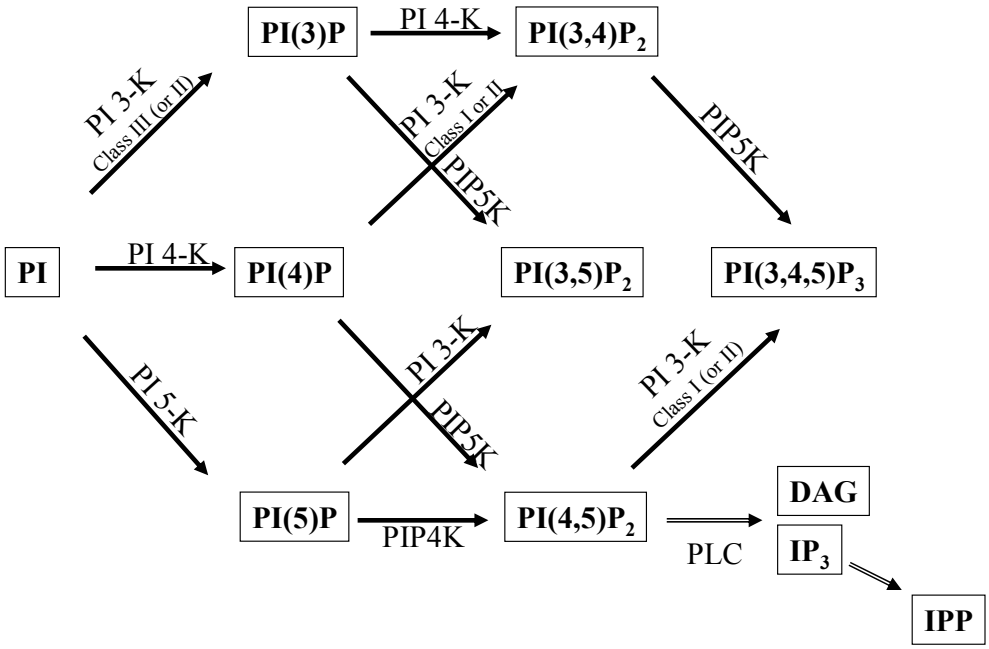

Figure 3 **Phosphoinositide synthesis in mammalian cells**.

Black arrow indicates production of phosphoinositide (phosphorylation by indicated kinases). In squares are the different phosphoinositide species. The phosphoinositide $PI(4,5)P_2$ can be further processed by phospholipase C (PLC) yielding DAG and IP_3. IP_3 can be further phosphorylated to produce inositol polyphosphates here refered as IPP. *Modified from (11)*

PTEN (for phosphatase and tensin homolog deleted on chromosome ten) is a $PI(3,4,5)P_3$ 3-phosphatase which was originally isolated as a tumour-suppressor gene in breast cancer and glioblastoma. There are some studies suggesting that PTEN also use inositol 1,3,4,5,6-pentakisphosphate (IP_5) as substrate and as well as being a protein phosphatase but the biological function remains unclear (47,48). The role of PTEN in the insulin signaling pathway is well characterized where the product of PI 3-K, $PI(3,4,5)P_3$, is dephosphorylated regenerating $PI(4,5)P_2$ (Figure 4). This results in the release of AKT from the plasma membrane which then is not able to perform its function (14,49).

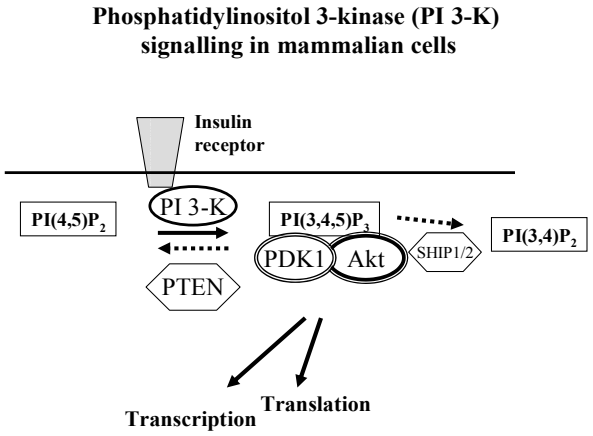

Figure 4 **The PI 3-kinase signaling cascade**.

Upon stimulation by growth factors such as insulin the tyrosine kinase receptor activates PI 3-K. $PI(4,5)P_2$ is converted to $PI(3,4,5)P_3$ by PI 3-K. This can be counteracted by the 3-phosphatase PTEN regenerating $PI(4,5)P_2$. The PH domain containing Akt and PDK1 are activated by $PI(3,4,5)P_3$ to further regulate transcription and translation. The $PI(3,4,5)P_3$ can be dephosphorylated by the 5-phosphatases SHIP1/2 at the 5' position to yield $PI(3,4)P_2$.

Different PTEN mutations or silencing have been implicated in various cancers probably due the hyperactivation of the insulin pathway (45:Vivanco, 2002 #265). This indicates the importance of proper regulation of the temporal lipid-protein interaction (47).

In addition to PTEN, the Src-homology-2 (SH2)-containing inositol 5-phosphatases, SHIP1 and SHIP2, dephosphorylate $PI(3,4,5)P_3$ produced by PI 3-K (insulin pathway) at the 5' position possibly yielding another potential second messenger $PI(3,4)P_2$ (47). SHIP1 is a hematopoetic-restricted enzyme and mutations in SHIP1 has been implicated in myeloid leukaemia (34). SHIP2 is ubiquitously expressed. However, mutations have primarily been implicated in genetic susceptibility to type 2 diabetes (34,50).

1.2.2 Regulation of PI(4,5)P_2 signaling by RHO GTPases and phosphatases, and the role of PI(4,5)P_2 in the regulation of the actin cytoskeleton and in membrane trafficking

PI(4,5)P_2 is mainly synthesized at the plasma membrane, accounts for approximately 1% of lipid molecules in the plasma membrane and is thus one of the more abundant PPI in the cell (10,51). The synthesis of PI(4,5)P_2 in mammalian cells is complex because it can be produced by either PI 5-P 4-kinase (PIP4K) or PI 4-P 5-kinase (PIP5K) (Figure 3). Furthermore they exist as different isoforms such as PIP5K α, –β and -γ as well as different splice variants (11:Yin, 2003 #99,52,53). However, PIP4K and PIP5K are not functionally redundant (24). The PIP4K is classified into PIP4K-α and PIP4K-β and are functionally non-redundant (11). PIP4K localizes to the cytosol, to the nucleus and to the endoplasmic reticulum but the physiological function of PIP4K is not well understood.

PIP5K variants also localizes to distinct cell compartments such as to the plasma membrane, at focal adhesions, to the nucleus and to the Golgi yielding different pools of PI(4,5)P_2 (53). PI(4,5)P_2 plays an important role in the regulation of the actin cytoskeleton but also in exocytosis, endocytosis and ion channel regulation (19,24,53).

In mammalian cells, most phosphoinositide species are not detectable in unstimulated cells but rapidly accumulate after stimulation with various agonists (44). in contrast, PI(4,5)P_2 is primarily maintained at relatively constant levels in cells but changes are observed upon specific stimulation (53-55). Upon postsynaptic activity PI(4,5)P_2 production is increased and has been linked to endocytosis at the presynaptic terminus (56). In mammalian cells, the RHO family of small GTPases are important for the regulation of PI(4,5)P_2 (18). PIP5K activity is induced by the RHO-GTPases Rac1, RhoA and ARFs involved in the organization of the actin cytoskeleton and in membrane trafficking (57-59).

Inactivation of PIP5K signaling has been suggested to be performed by casein kinase I-dependent phosphorylation that has been observed both in mammalian cells and in *Schizosaccharomyces pombe* (53). However, inhibition of PI(4,5)P_2 signaling is primarily mediated by specific PPI 5-phosphatases (14).

One important PI(4,5)P_2 5-phosphatase is the OCRL1 (oculocerebrorenal Lowe) protein Interestingly, OCRL1 contains a central phosphoinositide 5-phosphatase domain and a RHO-GAP homology domain that interacts with the RHO-GTPase RAC (60,61).

Mutations in OCRL1 have been found to cause the oculocerebrorenal syndrome of Lowe leading to defects in epithelial cells in lens and kidney, and to mental retardation (19,62). OCRL1 localizes to the trans-Golgi network (TGN) and possibly to the lysosomes (60,62). TGN is the major sorting site involved in protein transport to the apical or basolateral parts in epithelial cells. Mutations in OCRL1 gene, increasing $PI(4,5)P_2$, has been suggested to lead to defective Golgi trafficking and abnormal delivery of lysosomal enzymes causing symptoms observed in the disease of Lowe syndrome (34,63). Furthermore, OCRL1 mutant cells are affected in the distribution of actin–binding proteins and have decreased long actin stress fiber (61).

Synaptojanin 1 belongs to the family of $PI(4,5)P_2$ 5-phosphatases with a three-domain structure including a N-terminal domain with homology to the *Saccharomyces cerevisiae* protein SAC1, a catalytic domain and a C-terminal domain rich in proline (64). The regulation of $PI(4,5)P_2$ by synaptojanin has been implicated in rearrangements of actin filaments (65). In addition, synaptojanin 1 regulates the transport of clathrin-coated vesicles in neurons through the turnover of $PI(4,5)P_2$ (19,66). Synaptojanin 1 is recruited by endophilin to presynaptic terminals where it terminates the $PI(4,5)P_2$-dependent clathrin coating which is followed by internalisation of the vesicle and uncoating producing an early endosome (67-69). Mutations of synaptojanin 1 is lethal and the neurons of mutant animals contain an unusual high amount of clathrin-coated vesicles and actin filaments in the endocytic zone suggestive of an increased endocytosis probably due to increased levels of $PI(4,5)P_2$ (70). In contrast, overexpression reduces actin stress fibers (19). Synaptojanin 1 interacts with several proteins through distinct interaction modules. EPS15 (Epidermal growth factor pathway substrate 15) interacts via its EH-domain with the NPF motif of synaptojanin (71). EPS15 seems to play a role in endocytosis where EH-NPF interaction seems to be important for signaling networks (72). Synaptojanin also interacts, probably through the proline rich C-terminal domain, with the SH3 domain of adaptor protein GRB2 involved in the organization of the actin cytoskeleton (65,73,74).

The action of PIP5K and the phosphatases, OCRL1 and synaptojanin 1, are primarily regulating the organization of the actin cytoskeleton and membrane trafficking. Several components of these two cellular processes have been found to bind $PI(4,5)P_2$ regulating their localization or activity.

1.2.2.1 The regulation of the actin cytoskeleton by PI(4,5)P_2

In response to extracellular signals cells move, change shapes and translocate organelle with the help of a dynamic actin cytoskeleton (44). Actin remodelling is important for various processes such as the formation of filopodia, lamellipodia, membrane ruffles, phagosomes and endocytosis (24,53). Actin cytoskeleton remodelling is important in cell motility in different cell types such as neutrophils, macrophages, platelets and in the nervous system (53,75). The dynamic character is accomplished through the action of specific proteins and other molecules like PPI that regulate assembly/disassembly of the actin structure (18,44,76). Cytoskeletal proteins were one of the first shown targets of PPI binding and regulation. It was also observed that increased PI(4,5)P_2 synthesis initiates actin assembly and depletion of PI(4,5)P_2 triggers actin depolymerization (19).

Actin is present in the cell both as monomeric form, globular actin (G-actin) and as filamentous actin (F-actin). An important regulatory step is the inhibition of actin severing and capping. This inhibition promotes actin polymerization which is in part controlled by PI(4,5)P_2 (19). Proteins regulating actin dynamics can be organized in four groups.

The first group involves sequestration (binding) of G-actin by certain proteins such as profilin. It inhibits spontaneous nucleation of actin and polymerization of F-actin. The function of profilin is complex and different studies have suggested either inhibiting or stimulating roles (77). Profilin forms complex with ATP-G actin and can associate with the barbed end maybe speeding up the nucleation process (78). Actin will be fully incorporated (leading to assembly of actin filaments) when profilin dissociates from the barbed end. Increased amounts of PI(4,5)P_2 lead to extensive actin polymerisation (79). The mechanism by which PI(4,5)P_2 promotes actin polymerization could be the dissociation of the profilin-actin interaction shown in *in vitro* (19,34). Additionally, this PI(4,5)P_2 induced actin polymerization has been suggested to alter membrane ruffling. Intriguingly, the binding of PI(4,5)P_2 to profilin has also been suggested to inhibit hydrolysis of PI(4,5)P_2 by PLC (80).

The second group regulating actin dynamics comprises proteins involved in actin polymerization controlled by various reversibly capping proteins, gelsolin/villin family and CapZ family that bind the barbed ends of F-actin preventing elongation (81,82). Capping protein can also accelerate nucleation of new filaments from subunits by binding to small

oligomers. Rac and phosphoinositides regulate release of capping proteins (gelsolin/villin family of severing and/or capping proteins and the CapZ family or ADF/cofilin family proteins) from the barbed ends of actin filaments, allowing polymerization of the F-actin (82). Arp2/3 complex and associated factors seem to cap pointed ends and nucleate actin filaments of the sides of pre-existing filaments to form a branched network of actin important for cell motility (78).

The third group regulating actin dynamics consists of some ADF/cofilin and gelsolin family members that sever and/or cap actin filament and appear to accelerate depolymerization from the pointed ends (83). Cofilin binds Ca^{2+} independently of monomeric actin (G-actin) and filamentous actin (F-actin) in a 1:1 molar ratio. It severs and depolymerizes F-actin in a pH-dependent manner. $PI(4,5)P_2$ bind cofilin and thereby interfere with the interaction between actin and cofilin (84).

The fourth group regulating actin dynamics comprises proteins that crosslink actin filaments and the membrane. These interactions help linking the actin cytoskeleton to the plasma membrane by interacting with both actin and membrane proteins, such as integrins and receptors, and may help to direct signals to the actin cytoskeleton for remodelling. The crosslinking proteins such as α-actinin, ERM family members and talin have been shown to bind and be positively regulated by $PI(4,5)P_2$ (19,53).

1.2.2.2 The regulation of membrane trafficking by $PI(4,5)P_2$

In addition to the important role in actin cytoskeleton dynamics, $PI(4,5)P_2$ signaling plays a role in membrane trafficking. The release of neurotransmitters from neural and neuroendocrine cells is mediated by the exocytotic fusion of synaptic vesicles (SVs) and dense-core vesicles (DCVs) with the plasma membrane where $PI(4,5)P_2$ regulation is suggested to be an important factor (85). SVs transport non-peptide neurotransmitters such as glutamate and DCVs transport peptide neurotransmitters such as dopamine (67). Exocytosis is a regulated process including vesicle docking, priming and fusion (53). $PI(4,5)P_2$ has been suggested to be important for the ATP-dependent priming phase of exocytosis (6,86). $PI(4,5)P_2$ has been shown to bind to synaptotagmin, CAPS (calcium-dependent activator protein for secretion) and the MINT-proteins all involved in exocytosis. Synaptotagmin is proposed to regulate membrane traffic in neuronal and nonneuronal cells

through the docking step which would be regulated by the binding to $PI(4,5)P_2$ (87). CAPS is involved in a triggering step after docking and priming of vesicles (88).

Furthermore, $PI(4,5)P_2$ is an essential cofactor for clathrin-dependent endocytosis, phagocytosis, pinocytosis and endosome motility (67,89-91). In clathrin mediated endocytosis, $PI(4,5)P_2$ is required for the recruitment of adapter proteins such as AP-2 and AP180 suggesting that $PI(4,5)P_2$ could be a signal for endocytic localization (24,53,92,93). The recruitment of adaptor proteins is required for the invagination of the plasma membrane to form a clathrin-coated pit (24). The initiation of the clathrin-mediated endocytosis is suggested to be initiated by the GTPase ARF which is a probable activator of PIP5K (94). The generation of free vesicles from the clathrin-coated pits requires the PH-containing GTPase dynamin that mainly binds $PI(4,5)P_2$ (67). The termination of the $PI(4,5)P_2$ signaling is suggested to induce the uncoating of the clatrin vesicle (70). In phagocytosis $PI(4,5)P_2$ is required for local changes in actin dynamics that helps in the formation of the phagocytic cup (95).

1.2.3 Phospholipase C and inositol polyphosphates signaling

$PI(4,5)P_2$, in addition to functioning as a signaling molecule, can be processed further yielding another family of signaling molecules, the inositol polyphosphates (IPP) derived from the hydrolysis and phosphorylation by specific lipases and inositol phosphate kinases (14,48). The lipase hydrolysing $PI(4,5)P_2$ is known as phosholipase C (1-phosphatidylinositol-4,5-bisphophate phosphodiesterase 1) (Figure 3). Besides differential distribution, there are several isoforms of phosphoinositide-specific phospholipase C (PLC) such as PLC-β, -γ and -δ in mammalian cells (48,96). Most PLC isoforms contain a PH domain which is suggested to bring the enzyme to its substrate (48). All PLC hydrolyse $PI(4,5)P_2$ to yield inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a known Ca^{2+} channel receptor-binding second messenger. IP_3 induces the opening of the channels thereby increasing the cytosolic levels of Ca^{2+} released from intracellular stores (97). DAG is known to activate some isoforms of protein kinase C (PKC), a group of kinases involved in many different cellular processes, by binding to the C1 domain suggested to induce membrane interaction (98-100). DAG signaling can be negatively regulated by DAG kinases by converting DAG into phosphatidic acid (101). There is also

lipid signaling involving PLC function within the nucleus stimulated by growth hormones such as PDGF or IGF (48,102). A possible molecular mechanism is the activation of PKC isoforms by DAG and the subsequent phosphorylation of lamins that could induce nuclear disassembly (102).

Besides the role as second messenger, IP_3 can be further phosphorylated to yield different species of inositol polyphosphates involved in different signaling pathways (12,103). There are more than 60 inositol polyphosphates identified so far in different species. One of the first discovered was inositol-1,3,4,5-tetrakisphosphate (IP_4) which is produced by the phosphorylation of the 3' position by IP_3 3-kinase regulated by calmodulin (104). There are different isoforms of IP_3 3-kinase present in different cell types such as neurons, endothelial cells where $InsP_4$ is involved in the activation of Ca^{2+} signaling at the plasma membrane but has also been shown to be involved in T lymphocyte development (12,105).

1.2.4 The role of phosphoinositide in diseases

As described previously defects in phosphoinositide regulation, exemplified by the insulin signaling pathway, can lead to different diseases. In addition, phosphoinositide signaling can be used for defense against foreign organism or substance but host cell phosphoinositide signaling can also be used by pathogens for their own purpose.

Changes in phosphoinositide levels, specifically $PI(4,5)P_2$, have been observed during formation and internalization of the phagocytic cup in the phagocytic cells, macrophages (91). In another type of phagocytic cell, the neutrophil, the phagocyte NADPH oxidase involved in ROS is used to kill ingested microbes. To avoid injury to host tissues the assembling of the NADPH oxidase is regulated. A regulation mechanism is the migration of the oxidase to specific membrane compartments which is suggested to be partly controlled by PX-phosphoinositide interaction (106).

Some bacterial pathogens such as *Listeria monocytogenes* (causes meningitis) and *Shigella flexneri* (causes bacillary dysentery) have evolved to use the phosphoinositide signaling of the host for their own purpose to allow invasion through uptake (34,107). Infection with *Listeria* results in activation of the host PI 3-K which is suggested to induce a reorganization of the actin cytoskeleton facilitating uptake of pathogen by phagocytosis (108). Upon *Shigella* invasion one of the enzymes injected is the invasion plasmid gene D

(IpgD) that shows similarity to mammalian phosphoinositide 4-phosphatase and uses the host cells PI as substrate (109). IpgD has been implicated in the decreased cytoskeletal-membrane adhesion inducing membrane ruffles thereby enhancing bacterial uptake (110). A similar mechanism is observed in the bacterial pathogen *Salmonella typhimurium* where a *Salmonella* orthologue to IpgD, SigD (salmonella invasion gene), is important for host invasion (111). In the fungal pathogen *Candida albicans* a PIP5K was suggested to be involved in the morphogenetic process towards virulence (112).

1.2.5 Phosphoinositide biosynthesis in other organisms

In plants, phosphoinositide signaling is also including various PPI kinase isoforms and splice variants. The PPI signaling in plants have mainly been implicated in responses to abiotic stress such as drought and cold (48). In lower eukaryot phosphoinositide biosynthesis and signaling is also important as exemplified by PI 3-K signaling in the soil-living amoeba *Dictyostelium discoideum*. PI 3-K in *Dictyostelium* plays a role in cell movement in response to chemoattractant stimulation, chemotaxis. PI 3-K is suggested to control the reorganization of the actin cytoskeleton and redistribution of the subcellular localization of signaling components. Specific PH domain-containing proteins involved in chemotaxis bind to PI 3-K products and their localization is PI 3-K-dependent (113). PI 3-K signals to Akt that activates PAKa required for myosin II assembly during cytokinesis and chemotaxis where PTEN is responsible for the reverse regulation by dephosphorylation (113,114). In addition, there are four inositol 5-phosphatases in *Dictyostelium*. Each protein shows a distinct composition of domains beside the inositol 5-phosphatase catalytic domain suggesting different cellular roles (van Haastert 2003). In *Dictyostelium* phosphoinositide production has also been found to take place in the nucleus (115).

1.3 Phosphoinositide synthesis, turnover and function in *Saccharomyces cerevisiae*

In the model organism *Saccharomyces cerevisiae* phosphoinositide signaling is important involving several kinases and phosphatases (Figure 5)(14,116). These enzymes show in most cases specific kinase or phosphatase activity toward a particular phosphoinositide or PI and in addition be regulated by other proteins (14). There are two branches of phosphoinositide production in *Saccharomyces cerevisiae* with the final product PI(3,5)P_2 or PI(4,5)P_2 (Figure 5) (16). These two branches play a role in the regulation of the actin cytoskeleton and in membrane trafficking.

1.3.1 The role of PI(3)P and PI(3,5)P_2 signaling in membrane trafficking

In *Saccharomyces cerevisiae* the phosphatidylinositol 3-kinase VPS34 (vacuolar protein sorting) specifically converts PI to PI(3)P important for the carboxypeptidase Y (CPY) pathway including transport from trans-Golgi network (TGN), endosomal compartments and finally to the vacuole, the equivalent of the mammalian lysosome (117,118). VPS34 is similar to mammalian class III PI 3-K since it can only use PI as substrate (11). For activation, VPS34 is recruited by VPS15 to the Golgi/endosome (43,119,120). The VPS34-VPS15 complex also associates with the proteins VPS30 and VPS38 to form a multimeric complex, termed complex II (Figure 5) (121). In addition, VPS34 interacts with two proteins, APG6/VPS30 and APG14, that regulate autophagy therefore suggesting a role for PI(3)P in cytoplasm to vacuole transport (43). The phosphatidylinositol 3-phosphate 5-kinase FAB1 (yeast orthologue of mammalian PIKfyve) is non essential and is found on vacuolar membranes required for trafficking from the vacuole (122,123). Maximal synthesis of PI(3,5)P_2 by FAB1 requires VAC7 and VAC14 (124,125).

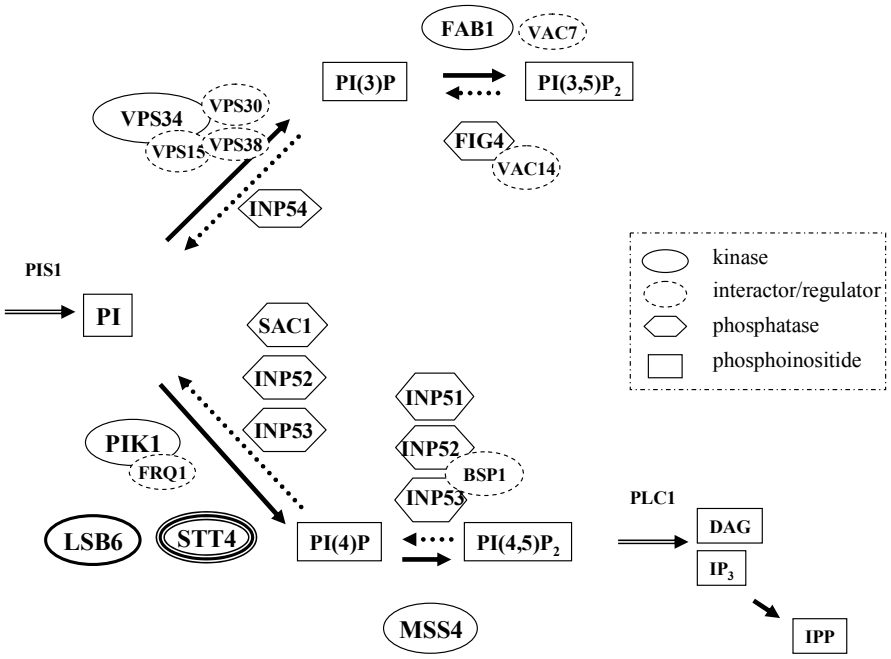

Figure 5 **Model of the synthesis and turnover of the known phosphoinositide species in *Saccharomyces cerevisiae*.** Solid arrow indicates synthesis of phosphoinositide and arrow with dots indicates turnover of phosphoinositide. In the case of PI(4)P it can be produces by three different PI 4-kinases. $PI(4,5)P_2$ can be further modified by hydrolysation by phospholipase C (PLC1) to yield DAG and IP_3. IP_3 can be further phosphorylated to yield inositol poly phosphates. Modified from. (16).

Loss of FAB1 function results in growth defect, an enlarged and poorly acidified vacuole, high temperature sensitivity and has defects in cargo selection for protein sorting within the multivesicular body sorting pathway. These phenotypes are also observed in *VAC7* or *VAC14* deletion mutants (122,124,126). $PI(3,5)P_2$ is present at low levels under vegetative growth but increases significantly upon osmotic stress suggesting a role in this particular stress response. This stress response does not involve the HOG1 (hyperosmotic stress response) pathway (127). For maximal activity during hyper-osmotic shock FAB1 requires the presence of VAC7 and VAC14 (128,129). Mutations in the 5-phosphatase *FIG4* were reported to suppress the vacuole size defect and temperature sensitivity of *vac7* by

increased PI(3,5)P_2 levels (130). FIG4 was originally identified in a screen for pheromone-regulated genes as an induced gene (14). FIG4 localizes to the vacuole, contains a SAC1 domain and mediates the turnover of PI(3,5)P_2 (Figure 5) (130). Additional studies showed that the FIG4 localization to the yeast vacuole membrane is mediated by the FAB1 regulator VAC14 (125).

1.3.2 PI(4)P and PI(4,5)P_2 signaling and the regulation of the actin cytoskeleton and membrane trafficking

In *Saccharomyces cerevisiae* three PI 4-kinases exists: PIK1, STT4, and the newly identified LSB6 (Figure 5) (14,131). Deletion of one kinase cannot be rescued by increased expression of the other suggesting different functions for each kinase (132).

The PI 4-kinase PIK1, is essential and involved in mating-pheromone signaling cascade, protein exocytosis, structure of the Golgi and cytoskeletal function {Garcia-Bustos, 1994 #150;Audhya, 2000 #14;Walch-Solimena, 1999 #145;Flanagan, 1993 #389}. PIK1 localizes to the Golgi and to the nucleus but the role of nuclear localization remains unclear (133,134). The activity and the localization of PIK1 is regulated by the small Ca^{2+}-binding protein frequenin (FRQ1), the yeast orthologue of mammalian frequenin involved in vesicle mediated neurotransmitter release (135,136).

The PI 4-kinase STT4 (Figure 5) (staurosporine- and temperature-sensitive), is essential, localizes to the plasma membrane and is involved the organization of the actin cytoskeleton, cell wall integrity and normal vacuolar morphology (120,132,137,138). PI(4)P produced by STT4 is used both as a substrate by MSS4 to yield PI(4,5)P_2 and is also an essential component involved in the transport of the essential aminophospholipid phosphatidylserine from the ER to the Golgi (132,139). The transmembrane protein SFK1 is required for the correct localization of STT4 to the plasma membrane (132). SAC1 is the phosphatase responsible for the dephosphorylation of STT4 derived PI(4)P. A mutation in the 4-phosphatase *SAC1* was originally isolated as a recessive suppressor of actin mutations. This suppression was suggested to be mediated by a STT4-dependent PI(4)P signaling to the actin cytoskeleton (140,141). SAC1 has also been postulated to act on a pool of PI(4)P in the yeast Golgi but the role of this turnover remains unclear (Figure 5) (14,141). Furthermore, SAC1 has been localized to the ER depending on the C-terminal

transmembrane domain and shown to be important for vacuolar morphology (141). Some groups have observed dephosphorylation of PI(3)P by SAC1 but the biological role is unknown (125,142). SAC1 exhibits *in vitro* phosphatase activity towards all monophosphorylated PIs but *in vivo* SAC1 is only able to use PI(4)P as substrate due to its localization to the ER (141). STT4 is localized to the plasma membrane and it is possible that the STT4-generated PI(4)P is transported to the SAC1-containing membranes with via an unknown mechanism (141). Mutations in *SAC1* lead to different phenotypes such as inositol auxotrophy and ATP transport deficiencies. A *sac1* mutant is also reported to suppress the lethality of SEC14-deficiency. SEC14 is responsible for establishing a critical phospholipid composition in Golgi membranes (143). A *sac1* mutant shows synthetic interaction with *mpk1* mutants, a cell wall integrity pathway component. The growth defect correlates with abnormal levels of the chitin synthase CHS2 caused by defective trafficking (144). Deletion of *SAC1* also causes the chitin synthase CHS3 to be erroneously transported to the vacuole instead of to the cell periphery (107,145).

PI(4)P synthesized by STT4 is used further by the only phosphatidyl 4-phosphate 5-kinase in *Saccharomyces cerevisiae*, MSS4 to produce PI(4,5)P_2. MSS4 was originally found as a multicopy suppressor of STT4 mutation and similar to STT4, MSS4 localizes to the plasma membrane (14,53,146,147). (Figure 5). MSS4 shows 40-44% identity to human PIP5K (11). In addition, conditional *mss4* mutant is functionally replaced by the murine type I β PI 4-P 5-kinase and the kinase activity is enhanced in the presence of phosphatidic acid which is characteristic of mammalian PIP5K (146,148). MSS4 is essential and required for the proper organization of the actin cytoskeleton and endocytosis (146,147). MSS4 has also been suggested to play a role in prospore formation being upstream of the phospholipase D (PLD), SPO14. SPO14-mediated PtdOH production is in accordance to PI(4,5)P_2 as cofactor of PLD in mammalian cells (149,150). In addition, an MSS4 mutant was found to rescue the growth defect of CSG2 (involved in sphingolipid synthesis) mutant induced by the presence of Ca^{2+}. In the same experiment *TOR2* and *AVO3* mutants, components of the TORC2 signaling pathway involved in the organization of the actin cytoskeleton, were also found among the suppressors. The link between MSS4, TORC2 and sphingolipid synthesis remains unclear (151).

Levels of PI(4,5)P_2 seems to be stable under vegetative growth but increases upon heat shock suggesting a regulation mechanism (147). MSS4 localization to the plasma

membrane is regulated by casein kinase I phosphorylation. The same study shows that MSS4 has a functional bipartite NLS. Overexpression of MSS4 leads to its partial localization to the nucleus suggesting a possible mechanism of regulation (152). However these two regulation mechanisms have not been linked to the role of MSS4 in the organization of the actin cytoskeleton.

Turnover of PI(4,5)P_2 in *Saccharomyces cerevisiae* is controlled by INP51, INP52, INP53 (Figure 5) that belongs to the phosphoinositide 5-phosphatase family (14,116). In contrast, INP51, INP52 and INP53 belong to the synaptojanin-like family that are highly conserved phosphoinositide 5-phosphatases defined by three domains: a Sac1-like domain exhibiting in most cases phosphoinositide phosphatase activity, a central PI(4,5)P_2 5-phosphatase domain, and a C-terminal proline-rich domain (14). Cells lacking both *INP51* and *INP52* (*inp51 inp52*) exhibits thickened cell wall, endocytosis defects, actin cytoskeletal disorganization and fragmented vacuolar structures (153,154). *inp51 inp53* double deletion mutant show synthetic interaction but not as severe as *inp51 inp52* (14). Interestingly, *inp53* deletion mutant, but not *inp52*, show compensatory mechanisms upon loss of SAC1 phoshatase activity (141). The triple *INP51*/*INP52*/*INP53* deletion mutant is not viable (154). INP52 and INP53 via the Sac1 domain interacts with the protein BSP1, which is suggested to be linked to the cortical actin cytoskeleton (155). Furthermore, INP53 has been implicated in the process of Golgi to endosomal trafficking and may localize to the Golgi (14). All these phenotypes indicate that INP51, INP52 and INP53 have overlapping functions as well as unique functions. INP52 and INP53 have the capacity to dephosphorylate all four phoshpoinositides observed in yeast in contrast to INP51 that only dephosphorylate PI(4,5)P_2 (14,125); (156). INP51 differs from INP52 and INP53 in that the Sac1-like domain lacks phosphatase activity which could explain the specific PI(4,5)P_2 5-phosphatase activity (157). *INP51* deletion mutant confers synthetic interaction with a *pan1* conditional mutant defective in endocytosis (158). PAN1 is the yeast orthologue of mammalian EPS15 (involved in clathrin-coated vesicle formation) known to interact with the mammalian orthologue of yeast INP family, synaptojanin 1 (see 1.3.2). The phenotypes of *inp51* mutant suggest that it has overlapping functions with INP52 and INP53 but that it has unique functions as well. It is suggested to be involved in endocytosis and regulation of the actin cytoskeleton during vegetative growth (14). It was observed that in some genetic backgrounds the *inp51* mutant shows cold resistance and simultaneous deletion of

PLC1 does not affect this tolerance. This indicates that PLC1 mediated hydrolysis of $PI(4,5)P_2$ is not required for INP51 signaling (156).

1.3.3 $PI(4,5)P_2$ targets in *Saccharomyces cerevisiae*

In *Saccharomyces cerevisiae* there are more than 20 proteins with known and unknown function that contains a putative PH domain that possibly can bind $PI(4,5)P_2$. The PH-containing proteins are implicated in different processes and localization differs widely suggesting that the function of PH domain is complex. There are also cellular processes affected by $PI(4,5)P_2$ biosynthesis but the targets are not known. $PI(4,5)P_2$ synthesis and turnover has been suggested to be important in vacuole dynamics such as the process of priming and docking important for vacuole association but the mechanism and targets remain unknown (23). There are several actin cytoskeleton regulating proteins that bind $PI(4,5)P_2$ but do not contain a specific domain such as profilin and twinfilin (159,160). Profilin localizes to the plasma membrane through electrostatic interactions with $PI(4,5)P_2$ and not via a PH domain (18,159). $PI(4,5)P_2$ could regulate actin polymerization via profilin indicated by the synthetic interaction observed in an *mss4* / *pfy1* double mutant (146). The phosphoinositide-binding proteins BOI1 and BOI2 are implicated in bud growth linked to RHO3 GTPase signaling (161,162). The RHO1-GEF ROM2 binds $PI(4,5)P_2$ through its PH domain which regulates its localization. PH domains are not only found in GEFs but also in GAPs such as BEM2 or BEM3 that also contains a PX domain. The binding to PPI has not been characterized (163).

There are other domains than PH domains that can bind $PI(4,5)P_2$ which is the case for ENTH domain. The mammalian epsin orthologue proteins in yeast, ENT1 and ENT2, contain an ENTH domain and might act as adaptors to recruit the machinery for actin polymerization and internalization of ubiquitinated receptor (164,165). SLA2, linked to RSP5 (ubiquitin-protein ligase) signaling, is another ENTH-containing protein involved in both endocytosis and the organization of the actin cytoskeleton (166).

There are several proteins that harbour a PH domain but do not localize to the plasma membrane to where $PI(4,5)P_2$ synthesis seems to be restricted (146,167,168). The oxysterol-binding proteins are examples of PH domain containing proteins that localize to the Golgi and are suggested to bind $PI(4,5)P_2$ (169,170).

1.3.4 Phospholipase C and inositol polyphosphate signaling

PLC1 (Figure 5) is the only phosphoinositide-specific phospholipase in *Saccharomyces cerevisiae* (171). ISC1 is another phospholipase C in *Saccharomyces cerevisiae*, but its substrate is inositol phosphosphingolipid (172). PLC1, most similar to mammalian PLC-δ isoforms, hydrolyzes $PI(4,5)P_2$ to yield diacylglycerol (DAG) and inositol 1,4,5-phosphate (IP_3) (48). PLC1 is important for cell growth since the loss of PLC1 function results in slow growth or is lethal depending on the genetic background of strains (173). Mutations in *PLC1* lead to many different phenotypes. It has been suggested to be required for a number of nutritional and stress-related responses such as glucose metabolism and oxidative stress (171,174-177). PLC1 has been suggested to be involved in nitrogen signaling pathway by interacting with GPR1 a hormone receptor-like plasma membrane protein (178). Furthermore, overexpression of *PLC1* is able to suppress defects in the TORC1 and TORC2 signaling pathway involving temporal control of cell growth and organization of the actin cytoskeleton respectively (Figure 7) (179). It is not clear how the products of PLC1, IP_3 and DAG are linked to these two signaling pathways. IP_3 has been shown to be involved in raising Ca^{2+} levels in the cell but the IP_3 target remains unknown (177). In *Saccharomyces cerevisiae* there is no known target for DAG but it has been suggested to be the protein kinase C, PKC1, involved in the organization of the actin cytoskeleton (180). PKC1, a component of the cell wall integrity pathway and the TORC2 signaling pathway, has a putative DAG binding domain (179,181-183).

The IP_3 produced by PLC1 can be further phosphorylated by different kinases to yield inositol polyphosphates (184). Inositol phosphate kinase 2 (IPK2) and inositol phosphate kinase 1 (IPK1) convert IP_3 to inositol-1,3,4,5,6-pentakisphosphate (IP_5) and inositol-1,3,4,5,6-hexakisphosphate (IP_6) respectively (185,186). Inositol polyphosphates have been implicated in nuclear signaling (102). In *Saccharomyces cerevisiae* ARG82/IPK2, an inositol phosphate kinase, is needed for efficient recruitment and regulation of chromatin remodelling complexes to the PHO5 promoter where PHO5 is involved in phosphate metabolism (17). Another inositol phosphate kinase, KCS1, can further pyrophosphorylate some of the IP_6 to yield bisphosphoinositol-IP_5 (PP-IP_5) which has been implicated in biogenesis of the vacuole and response to environmental stress (187,188).

1.4 The cell wall integrity pathway and the TOR complex 2 signaling pathway

Different signaling pathways in *Saccharomyces cerevisiae* regulate cell growth such as by nutrient availability sensing by the TORC1 signaling pathway. Growth is oriented by a polarized actin cytoskeleton that directs secretory vesicles to the growth site (189). The actin cytoskeleton is important for the transport of cell wall components. The cell wall is a complex network of molecules and proteins that surrounds the entire cell. Furthermore it is important for the rigidity of the cell preserving the osmotic integrity (190). Remodeling of the cell wall is important during vegetative growth and during pheromone-induced morphogenesis (191). In addition, the yeast cell wall is important for cell survival in a fluctuating environment (192).

1.4.1 The actin cytoskeleton

The actin cytoskeleton of *Saccharomyces cerevisiae* is built up of cables and patches. Cables are filamentous actin that organizes in parallel towards the incipient and growing bud. Actin patches consist of actin and actin-binding proteins that localize to the incipient and growing bud. Both cables and patches change their arrangement in a cell cycle-dependent manner (193). There are many actin-binding proteins which are conserved from yeast to mammals such as twinfilin and profilin that are regulated by PPI (see 1.3.3.1) (159,194). The actin cytoskeleton is important for the cell shape, is highly dynamic in response to changes in the external environment and implicated in different cellular processes such as endocytosis (76,195,196).

1.4.2 The cell wall

The cell wall is composed of an outer layer of glucan (60%), mannoproteins (40%) and a small amount of chitin (1%). Glucan, produced by FKS1 an FKS2, gives rigidity to the cell and the synthesis is cell-cycle dependent. The mannoproteins (cell wall proteins) are found on the outside of the cell wall and are linked via β1,6-glucan to the underlying β1,3-glucan (190). Chitin (polymer of N-acetylglucosamine) synthesis is cell-cycle dependent and is

deposited (below glucan) to specific locations of the cell such as a ring at the neck of the emerging and growing bud (144,197). Chitin synthesis involves three enzymes: CHS1, CHS2 and CHS3 that are tightly regulated (190,198). CHS1 is involved in cell wall repair (197). CHS2 is required for the formation of the chitin disk that closes the primary septum at cytokinesis (199). The function of CHS3 is to produce the chitin ring that appears at the basis of an emerging bud and of the small amount of chitin interdispersed in the cell wall and is therefore the major chitin producing synthase (90% of total chitin) (197). The regulation of chitin synthesis is complex including transcriptional and posttranslational control (191,200). Transcription seems to be regulated at different stages of the cell cycle. CHS3 is posttranslationally modified and regulated by different mechanisms: its localization/transport is regulated by the actin cytoskeleton and endocytosis, polarity establishment proteins, PKC1 pathway and specific chitin synthase proteins such as CHS5 and CHS7 (197,199-201). Upon (genetic) disturbance of the glucan composition of the cell wall it is postulated that a compensatory mechanism is activated by increased chitin production indicating tightly controlled cell wall structure (192).

1.4.3 The cell wall integrity pathway

Response to cell wall damage to prevent the cell from cell lysis includes four mechanisms: changes in the level of cell wall components such as chitin, the association between the cell wall components, a transient redistribution of cell wall synthesis and a transient depolarization of the actin cytoskeleton probably redirecting the cell wall machinery (197,202). Normally, cell wall synthesis occurs at the active growth site but upon damage cell wall synthesis redistributes to the whole cell periphery (195,203). The cell wall integrity pathway has been suggested to sense and activated by cell wall damage induced by heat or Cell wall disturbance upon heat or hypotonic shock. Activation of the cell wall integrity pathway induces the expression of genes important for the cell wall synthesis or assembly to repair cell wall damage (192).

Cell wall stress is monitored by transmembrane proteins including members of the WSC family and the MID2 protein (195,202). Cell wall damage probably induces membrane stretch that is sensed by these integral membrane proteins (204-206). Similar to mammalian cells, RHO GTPases in *Saccharomyces cerevisiae* are important for the

organization of the actin cytoskeleton (207). In *Saccharomyces cerevisiae* the RHO GTPases RHO1 (Figure 6) and possibly RHO2 control polarization of the actin cytoskeleton and cell wall synthesis thereby mediating bud growth and regenerating the cell wall upon damage (197,208,209). RHO1 activity is regulated by a set of proteins: GDP/GTP exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). WSC1 transduce response to cell wall damage by activating RHO1 via the GEF ROM2 by an unknown mechanism (195,204). ROM1 and TUS1, homologous to ROM2, are also involved in the activation of RHO1 (210,211). The GAP SAC7, an important negative regulator of RHO1 and negative regulator of the cell wall integrity pathway, transforms RHO1 into an inactive GDP-bound form by increasing RHO1's low intrinsic GTPase activity. BEM2, BAG7 and LRG1 are also RHO1 GAPs regulating different RHO1 functions (163,212,213). BEM2 and SAC7 negatively control the actin cytoskeleton through the PKC1 pathway. In addition, SAC7 and BAG7 also negatively control the actin cytoskeleton by an unknown effector pathway. LRG1 negatively regulates the RHO1-dependent activation of FKS1 but does not regulate the PKC1-MAP kinase cascade (212).

The existence of different functions of the GAPs probably ensures regulation of the effector pathways at the appropriate place and time (163). It is not clear how the GAPs achieve their specificity but differential localization and regulation is a possible mechanism which is exemplified by BEM2 that contains a PH domain (163).

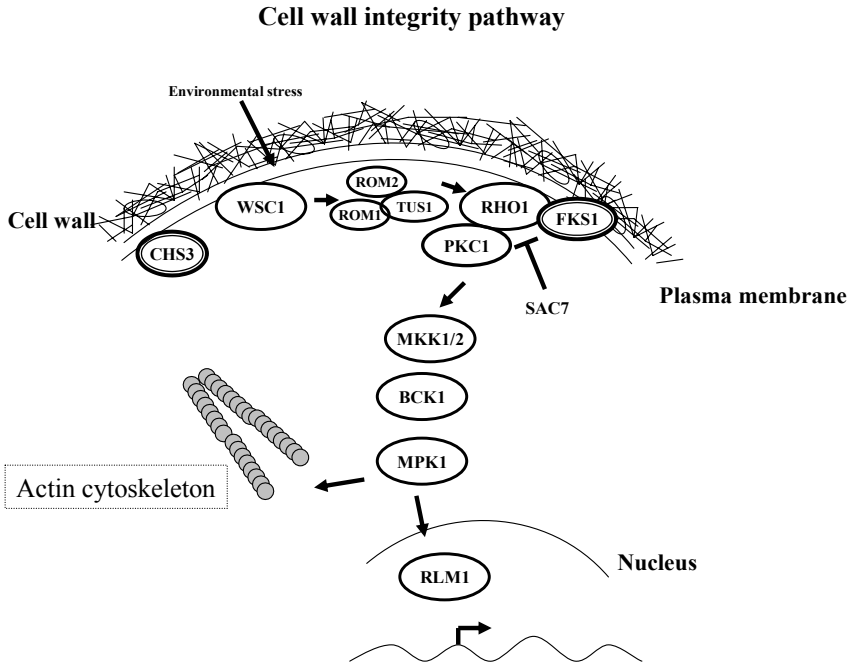

Figure 6 **The cell wall integrity pathway**

Environmental stimuli that affect cell wall is sensed by WSC1 that activates the effectors of the cell wall integrity pathway. This induces reorganization of the actin cytoskeleton and changes in transcription of genes involved in the remodelling of cell wall.

RHO1 has five downstream effectors (163). RHO1 is part of the β 1,3-glucan synthase complex comprising FKS1 or FKS2 (depending on environmental conditions) which is stimulated in a GTP-dependent manner (190,214). RHO1 regulates the glucan synthase complex through the localization and activity of FKS1 and FKS2 (195,208). In addition, RHO1 also interacts with a formin-family member BNI1, with the stress responsive transcription factor SKN7 and with SEC3 involved in polarized exocytosis (215-218). The fifth effector is PKC1, component of the cell wall integrity pathway, is activated by RHO1 leading in turn to the activation of a MAP (mitogen activated) kinase cascade. This MAP kinase cascade consisting of BCK1, MKK1/2 and MPK1 (Figure 6) (181,195,219,220). PKC1 is activated upon cell wall stress such as heat shock, to control the organization of the actin cytoskeleton and transcription of genes encoding cell wall biosynthetic enzymes (179,221,222). RHO1 and PKC1 are needed both for depolarization and repolarization of

the actin cytoskeleton (195). It has been shown that PKC1 is activated by phosphatidylserine *in vitro* that is characteristic for the atypical ζ isoform of protein kinase C in mammalian cells. It has been suggested that PKC1 interacts with lipids (diacylglycerol) via its C1 and/or C2 domain but the physiological role remains unclear (220,223).

The MAP kinase MPK1, downstream of PKC1, is activated by dual phosphorylation of its two conserved theronine (Thr^{190}) and tyrosine (Tyr^{192}) residues (224). The activation of MPK1 induces the transcription of genes involved in the cell wall biosynthesis such as CHS3 and integral cell wall proteins such as GPI proteins (191). The main transcription factor involved in the MPK1 signaling is RLM1 but other transcription factor might also be involved such as NHP6A and NHP6B and SBF (225-227). MPK1 interacts with RLM1 possibly for activation by phosphorylation (228,229). However, loss of MPK1 function leads to more severe phenotypic defects than a *rlm1* deletion mutant suggesting that *MPK1* have additional effectors (191).

1.4.4 The TOR signaling pathways

In *Saccharomyces cerevisiae,* the target of rapamycin proteins, TOR1 and TOR2, play an important role in the regulation of cell growth (Figure 7) and have strong similarity to the catalytic domains of PI 3-K and PI 4-K (11,230). This catalytic domain, named PIK (phosphatidylinositol kinase) domain, is present in various kinases, in addition to the TOR proteins, for example mammalian ATM (ataxia telangiectasia mutated) and the yeast MEC1 both involved in DNA damage response (230,231). However, it remains unclear if the PIK-domain has lipid kinase activity (230).

The TOR proteins in *Saccharomyces cerevisiae* are part of two distinct complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1, contains TOR2 or TOR1, and associated partners, is essential for temporal control of cell growth and controls transcription and translation in response to nutrients. TORC2 contains TOR2, but not TOR1, and associated partners AVO1, AVO2, AVO3 and LST8 and is essential for spatial control of cell growth through the regulation of the organization of the actin cytoskeleton (Figure 7) (232). TORC2 signals to the GEF ROM2 by an unknown mechanism activating a RHO1-dependent effector branch, the MAP kinase cascade, regulating the organization of

the actin cytoskeleton but not the cell wall synthesis (179,209,232). TOR proteins are phosphatidylinositol kinase-related kinase, as mentioned above, but so far it has not been shown to have lipid kinase activity. However, lipid signaling might be important in the TOR2 signaling cascade since it was observed that overexpression of *MSS4* or *PLC1* the growth defect of a *tor2* conditional mutant defective in the organization of the actin cytoskeleton (179). These results suggest that phosphoinositide signaling could have an important role in the TOR2 signaling pathway (179). ROM2 contains a PH domain which binds $PI(4,5)P_2$ (132,209). The localization of ROM2 changes in a *mss4* conditional mutant under non-permisssive conditions suggesting that $PI(4,5)P_2$ signaling is important for correct localization of ROM2 (132). In addition, overexpression of ROM2 lacking the PH domain is not longer able to suppress the growth defects of a *tor2* mutant. This suggests that the GEF ROM2 might be the link between MSS4 signaling and the TORC2 signaling pathway (209).

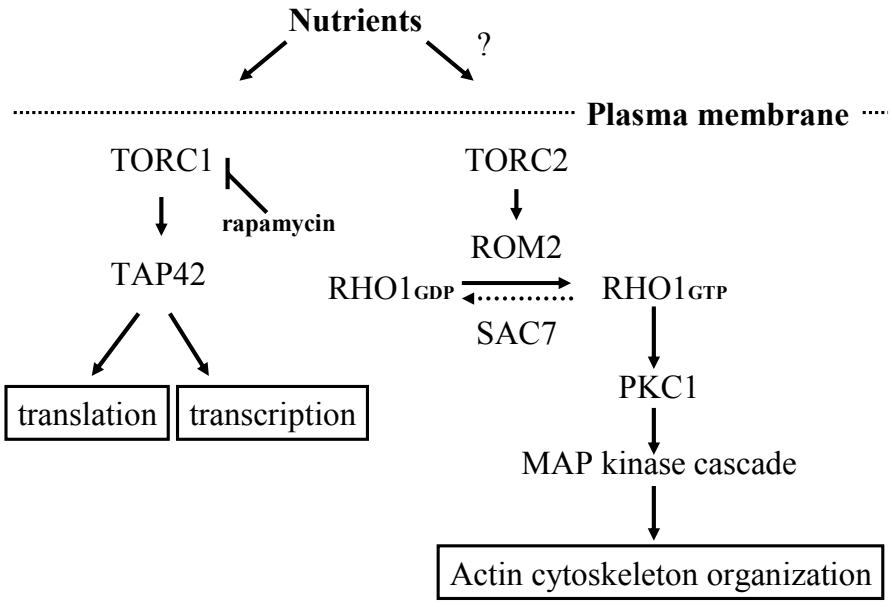

Figure 7 **The TOR signaling pathways**

TORC1 comprises either TOR1 or TOR2 and is important for temporal control of cell growth in response to nutrients regulating transcription and translation. TORC1 signaling can be inhibited by rapamycin. TORC2 only comprises TOR2, signals through the PKC1 pathway and is important for the organization of the actin cytoskeleton.

Aim of the thesis

The family of phosphoinositides, conserved from yeast to mammals, is a complex group of signaling molecules. The importance of phosphoinositide signaling is based on their implications in several human diseases.

The aim of this thesis is to understand how the essential phosphoinositide phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$), is regulated in the model organism *Saccharomyces cerevisiae.* We specifically focus on $PI(4,5)P_2$ signaling linked to the TORC2 signaling pathway and the cell wall integrity pathway studying the role of $PI(4,5)P_2$ 5-phosphatases INP51, INP52 and INP53 and how they are regulated.

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Calmodulin controls organization of the actin cytoskeleton via regulation of phosphatidylinositol (4,5)-bisphosphate synthesis in *Saccharomyces cerevisiae*

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In this article, Helena Morales-Johansson did the experiment presented in Fig 2

ABSTRACT

PPI regulate a wide range of cellular processes, including proliferation, survival, cytoskeleton remodelling and membrane trafficking, yet the mechanisms controlling the kinases, phosphatases and lipases that modulate phosphoinositide levels are poorly understood. In the present study, we describe a mechanism controlling MSS4, the sole phosphatidylinositol (4)-phosphate 5-kinase in *Saccharomyces cerevisiae*. Mutations in *MSS4* and *CMD1*, encoding the small Ca^{2+}-binding protein calmodulin, confer similar phenotypes, including loss of viability and defects in endocytosis and in organization of the actin cytoskeleton. Overexpression of *MSS4* suppresses the growth and actin defects of *cmd1-226*, a temperature-sensitive calmodulin mutant which is defective in the organization of the actin cytoskeleton. Finally, the *cmd1-226* mutant exhibits reduced levels of phosphatidylinositol (4,5)-bisphosphate. These findings suggest that calmodulin positively controls MSS4 activity and thereby the actin cytoskeleton.

Key words: CMD1, MSS4, PtdIns(4)*P* 5-kinase, PtdIns(4,5)P_2, *Saccharomyces cerevisiae*.

Abbreviations used: ARF, ADP-ribosylation factor; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PLC, phospholipase C; SD, yeast nitrogen base/ammonium sulphate/dextrose media; TRITC, tetramethylrhodamine β-isothiocyanate; ts, temperature-sensitive; YPD, yeast extract/peptone/dextrose media.

INTRODUCTION

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] plays a role in a remarkable number of cellular processes. PtdIns(4,5)P_2 is cleaved by phospholipase C (PLC) to generate the second messengers diacylglycerol and Ins(1,4,5)P_3 that activate protein kinase C (PKC) and increase intracellular Ca^{2+} respectively (233). Uncleaved PtdIns(4,5)P_2 directly binds and regulates several target proteins (for review see (6)). For example, PtdIns(4,5)P_2 (i) binds pleckstrin homology domains present in a variety of proteins involved in signal transduction (25,234,235); (ii) binds actin-binding proteins and thereby regulates organization of the actin cytoskeleton (6); (iii) regulates the small GTPase ADP-ribosylation factor (ARF) by recruiting both the guanine nucleotide exchange factor and the GTPase-activating protein for ARF ((236-238), but see (239)); and (iv) binds several proteins involved in the formation of clathrin-coated vesicles during endocytosis (240-242).

In mammalian cells, PtdIns(4,5)P_2 is synthesized via two independent reactions. It is the product of PtdIns(4)*P* 5-kinases, which phosphorylate PtdIns4*P* at the D-5 position of the inositol ring. It is also produced by phosphorylation of PtdIns5*P* at the D-4 position of the inositol ring by PtdIns(5)*P* 4-kinases (52). Little is known about the regulation of PtdIns(4,5)P_2 synthesis. The activation of PtdIns(4)*P* 5-kinases by phosphatidic acid, a product of phospholipase D, is well documented 13 (148,243,244), but two recent studies (245,246) demonstrate that PtdIns(4)*P* 5-kinases are also activated by ARF to control membrane ruffling and structural integrity of the Golgi complex. The investigation of the physiological function of PtdIns(4,5)P_2 and the mechanisms regulating its synthesis in mammalian cells is complicated not only due to the existence of both PtdIns(4)*P* 5-kinases and PtdIns(5)*P* 4-kinases, but also because of the presence of different isoforms of these kinases. In the yeast *Saccharomyces cerevisiae*, PtdIns(4,5)P_2 is produced solely via MSS4-mediated phosphorylation of PtdIns4*P* (147). MSS4 is encoded by the essential gene *MSS4* which when mutated causes disorganization of the actin cytoskeleton, aberrant cell morphology and loss of cell integrity (146,147).

Calmodulin is a Ca^{2+}-binding protein also implicated in the regulation of many proteins, including metabolic enzymes, protein kinases, a protein phosphatase, transcription factors,

ions transporters, receptors, motor proteins and cytoskeletal components (for reviews see (247,248)). Studies using temperature-sensitive (ts) calmodulin mutations (*cmd1*^{ts}) in *S. cerevisiae* have demonstrated that calmodulin is an essential protein required for organization of the actin cytoskeleton, endocytosis, nuclear division and bud emergence (249,250). Interaction of calmodulin with some target proteins has been reported (251,252); however, the exact mechanisms by which calmodulin controls these cellular processes is largely unknown. Our findings reveal a new mode of calmodulin action which operates via regulation of PtdIns(4,5)P_2 synthesis.

EXPERIMENTAL

Strains, plasmids and media

The *S. cerevisiae* strains used in this study are listed in Table 1. All strains were isogenic derivatives of JK9-3d. Plasmids used in this work are listed in Table 2. Rich media, YPD [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose], and synthetic defined minimal media, SD [0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulphate and 2% (w/v) dextrose], supplemented with the appropriate nutrients for plasmid maintenance were as described previously (253).

Table 1 Yeast strains

Strain	Genotype
JK9-3d**a**	MAT**a** *leu2-3,112 ura3-52 trp1 his4 rme1 HMLa*
JK9-3dα	MATα *leu2-3,112 ura3-52 trp1 his4 rme1 HMLa*
TB50**a**	JK9-3d**a** *his3 HIS4*
SD18-1d	TB50**a** *mss4*::*HIS3MX6*/Ycplac111::*MSS4*
SD19-3a	TB50**a** *mss4*::*HIS3MX6*/Ycplac111::*mss4-2^{ts}*
SD54-11d	SD18-1d *bar1::kanMX4*
SD55-1b	SD19-3a *bar1::kanMX4*
TS45-1a	TB50**a** *mpk1::TRP1*
SH121	JK9-3d**a** *ade2 tor2*::*ADE2-3*/Ycplac111::*tor2-21^{ts}*
SH27-1b	JK9-3dα *ade2 mpk1*::*TRP1 tor2*::*ADE2-3*/Ycplac111::*tor2-21^{ts}*
DBY7462	MAT**a** *leu2-3,112 ura3-52 trp1 his3 lys2*
DBY7446	DBY7462 *cmd1-226*
DBY7445	DBY7462 *cmd1-228*
DBY7449	DBY7462 *cmd1-239*

Table 2 Plasmids

Plasmid	Description
pROM2	pAS30, *ROM2* (2μ, *URA3*) (209)
pRHO2	pC-186, *RHO2* (2μ, *URA3*) (254)
pCMD1	pSD14, *CMD1* in YEplac195 (2μ, *URA3*)
pBCK1-20	*BCK1-20* in pRS352 (*CEN*, *URA3*) (183)
pMKK1	*MKK1* in YEp352 (2μ, *URA3*) (183)
pMPK1	*MPK1* in YEp352 (2μ, *URA3*) (183)
pMSS4	pSH22, *MSS4* in pSEY18 (2μ, *URA3*) (179)

pPKC1 pSH24, *PKC1* in pSEY18 (2μ, *URA3*) (179)

Determination of cellular phosphatidylinositol levels

Cells were grown at 24°C in the presence of 50μCi/ml [^{3}H]inositol (Amersham International, Little Chalfont, Bucks., U.K.) to a D_{600} of 0.7 (*D* is attenuance), harvested by centrifugation, and resuspended in one-fifth the original volume. After control samples had been removed, cells were incubated at 37°C and aliquots (200μl) were collected at different times. Cells were arrested by the addition of 2.5 vols. of methanol, and disrupted by vortexing for 5min with glass beads. Lipids were extracted and analysed as described previously (255). The radioactivity associated with each phospholipid was quantified and corrected to the level of total phosphatidylinositol found in the control samples.

Endocytosis assays

Lucifer Yellow (Fluka, Buchs, Switzerland) accumulation in vacuoles was assayed as described previously (256). Cells were grown in YPD at 24°C to mid-log phase, shifted to 37°C and incubated for 1h in the presence of 4mg/ml Lucifer Yellow. Cells were then washed three times in 1ml of ice-cold buffer (50mM sodium phosphate, 20mM sodium azide and 20mM sodium fluoride, pH7.0). [^{35}S]α-Factor-uptake assays were performed using the continuous-presence protocol as described previously (256). Cells were grown in YPD at 24°C to exponential phase, and the α-factor-uptake assay was carried out at 24°C or 37°C. Cells assayed at the restrictive temperature (37°C) were preincubated at this temperature for 15min.

Rhodamine–phalloidin staining of actin

Cells were grown to early logarithmic phase, fixed in formaldehyde, and stained with phalloidin–tetramethylrhodamine β-isothiocyanate (TRITC) conjugate (Sigma) to visualize actin as described previously (257).

RESULTS

Calmodulin and MSS4 are functionally related

We have shown previously that the yeast PtdIns(4)*P* 5-kinase MSS4 is an essential protein required for proper organization of the actin cytoskeleton (147). The actin cytoskeleton plays a fundamental role in endocytic uptake in yeast (258) and recent studies (259,260) in mammalian cells suggest a role for PtdIns(4,5)P_2 in receptor-mediated endocytosis. To investigate further the role of PtdIns(4,5)P_2 in endocytosis, we examined fluid-phase and receptor-mediated endocytosis in a temperature-sensitive (ts) *mss4* mutant. *mss4*^{ts} cells shifted to non-permissive temperature (37°C) were defective in fluid-phase endocytosis, as evidenced by a lack of vacuolar accumulation of the dye Lucifer Yellow, and in receptor-mediated uptake, as evidenced by a reduction in internalization of the α-factor pheromone (Figure 1). These results suggest that PtdIns(4,5)P_2 is required for the internalization step of endocytosis.

The above finding revealed that the phenotype of a *mss4*^{ts} mutant is remarkably similar to that of a *cmd1* mutant defective in the Ca^{2+}-binding protein calmodulin. Calmodulin, like MSS4, is an essential protein required for proper organization of the actin cytoskeleton (249) and for the internalization step of endocytosis (250,251). Furthermore, like a *mss4*^{ts} mutant, the temperature-sensitive *cmd1-226* mutant loses viability after prolonged incubation at restrictive temperature; approx. 30% of *cmd1-226* cells and 80% of *mss4*^{ts} cells were non-viable after 4h at 37°C, as evidenced by staining with the vital dye Methylene Blue. No loss of viability was detected with a similarly treated wild-type strain. The growth defect of both the *mss4*^{ts} and *cmd1-226* mutants was at least partly suppressed by osmotic stabilization of the cells, i.e. by the addition of 1M sorbitol to the growth medium (results not shown), indicating that both mutants are impaired in cell integrity. Finally, both *mss4* (146) and *cmd1-226* (Figure 2) mutants display aberrant morphology at 37°C. Altogether, these findings suggest that MSS4 and calmodulin have related functions.

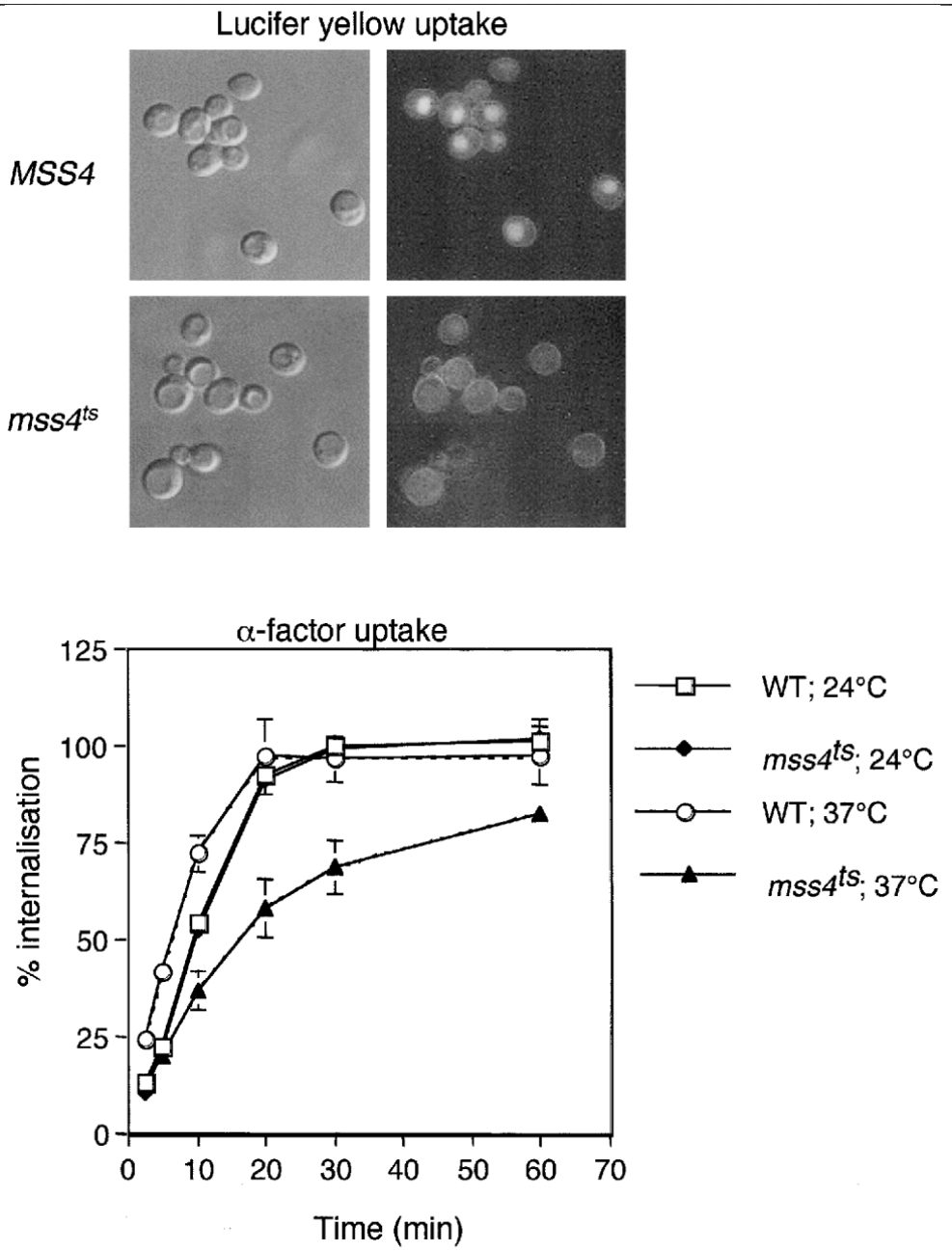

Figure 1 MSS4 is required for the internalization step of endocytosis

Wild-type (WT; *MSS4*; SD54-11d) and *mss4*^{ts} (SD55-1b) cells were assayed for Lucifer Yellow accumulation in the vacuole at 37°C (upper panel) or α-factor uptake at 24°C and 37°C (lower panel). For the Lucifer Yellow uptake, the same field of cells is viewed by fluorescence (right-hand panels) and Nomarski (left-hand panels) microscopy. The results shown for α-factor uptake represent the mean of two independent experiments±S.E.M.

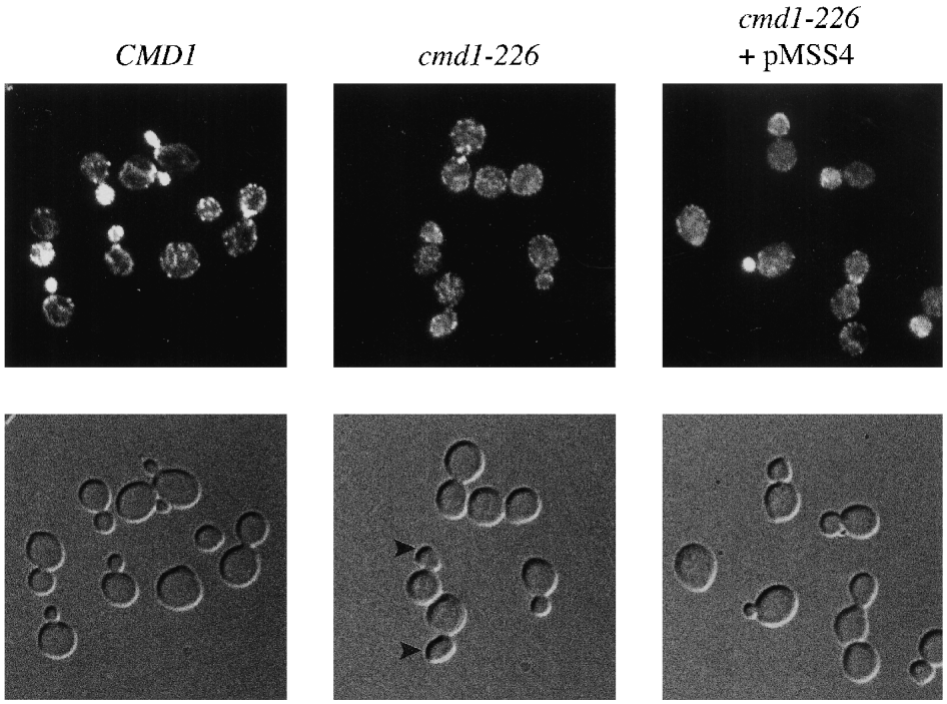

Figure 2 Multicopy *MSS4* suppresses a *cmd1* mutation

Logarithmic cultures of *cmd1-226* (DBY7446) cells carrying an empty vector (*cmd1-226*), pCMD1 (*CMD1*) or pMSS4 (*cmd1-226*+pMSS4) were grown at 24°C, shifted to 37°C for 4h, fixed, stained with TRITC–phalloidin, and observed by Nomarski (lower panel) and fluorescence (actin staining; upper panel) microscopy. Arrowheads indicate the abnormally-shaped, medium-sized buds of *cmd1-226* cells.

To investigate further whether calmodulin and MSS4 are functionally related, we examined whether *CMD1* and *MSS4* interact genetically. Temperature-sensitive *CMD1* mutations fall into different intragenic complementation groups, indicating a role for calmodulin in different, essential cellular processes (249,261). Representative mutations from the different complementation groups were examined for suppression by a multicopy *MSS4* gene. Overexpression of MSS4 restored growth (Figure 3) in a *cmd1* mutant defective in organization of the actin cytoskeleton (*cmd1-226*), but did not restore growth of mutants defective in calmodulin localization (*cmd1-228*) or in nuclear division (*cmd1-239*) (results not shown). The suppression of the *cmd1-226* growth defect by overexpression of MSS4 suggested that *MSS4* can restore proper organization of the actin cytoskeleton in *cmd1-226* cells. To test this, the actin cytoskeleton was visualized in *cmd1-226* mutant cells

overexpressing MSS4 (Figure 2). Cells were grown at permissive temperature (24°C) and then shifted to restrictive temperature (37°C) for 4h. *cmd1-226* cells exhibited a severe defect in the organization of the actin cytoskeleton; actin cables were undetectable and cortical actin patches were randomly distributed in mother and daughter cells, instead of being concentrated at the site of growth. Conversely, *cmd1-226* cells overexpressing MSS4 (*cmd1-226+pMSS4*) exhibited a polarized actin cytoskeleton, as observed in wild-type *CMD1* cells (Figure 2). Overexpression of *MSS4* also suppressed the abnormal morphology of medium-sized buds on *cmd1-226* mutant cells (Figure 2). Thus, consistent with the suppression of the *cmd1-226* growth defect by *MSS4*, overexpression of MSS4 suppresses the actin defect of *cmd1-226* cells.

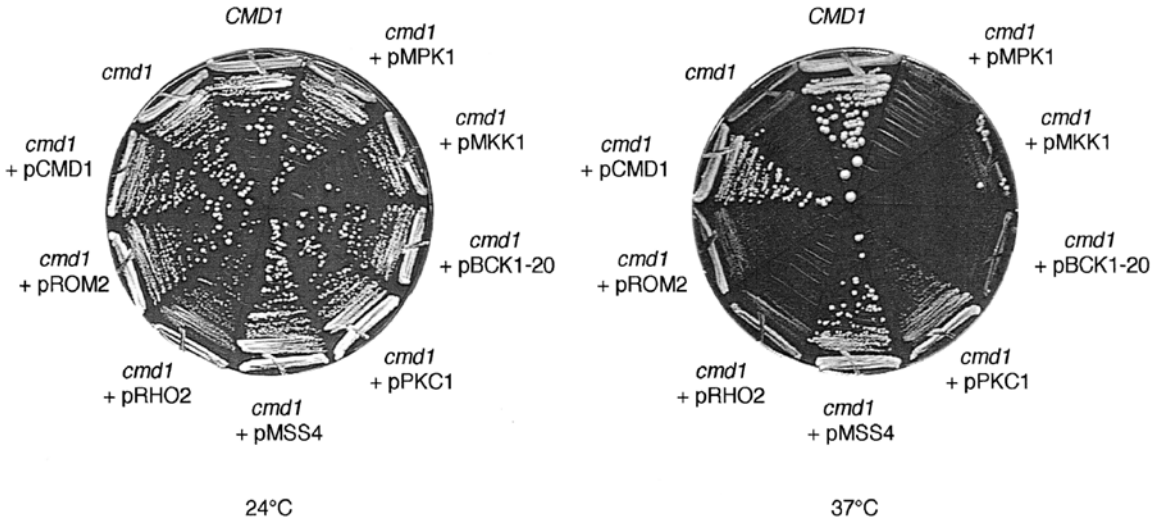

Figure 3 Overexpression of PKC1, but not other members of the PKC1-activated MAPK pathway, suppresses a *cmd1* mutation

Wild-type (*CMD1*; DBY7462) cells and *cmd1-226* (*cmd1*; DBY7446) cells carrying an empty vector or pCMD1, pROM2, pRHO2, pMSS4, pPKC1, pBCK1-20 (containing an activated allele of *BCK*1), pMKK1 or pMPK1 were streaked out on YPD and incubated at 24°C or at 37°C.

These findings indicate that calmodulin and MSS4 are functionally related, particularly in the regulation of the organization of the actin cytoskeleton. Furthermore, the above findings, combined with the observation that multicopy *CMD1* failed to restore growth in a $mss4^{ts}$ mutant (results not shown), suggest that calmodulin may act upstream of MSS4.

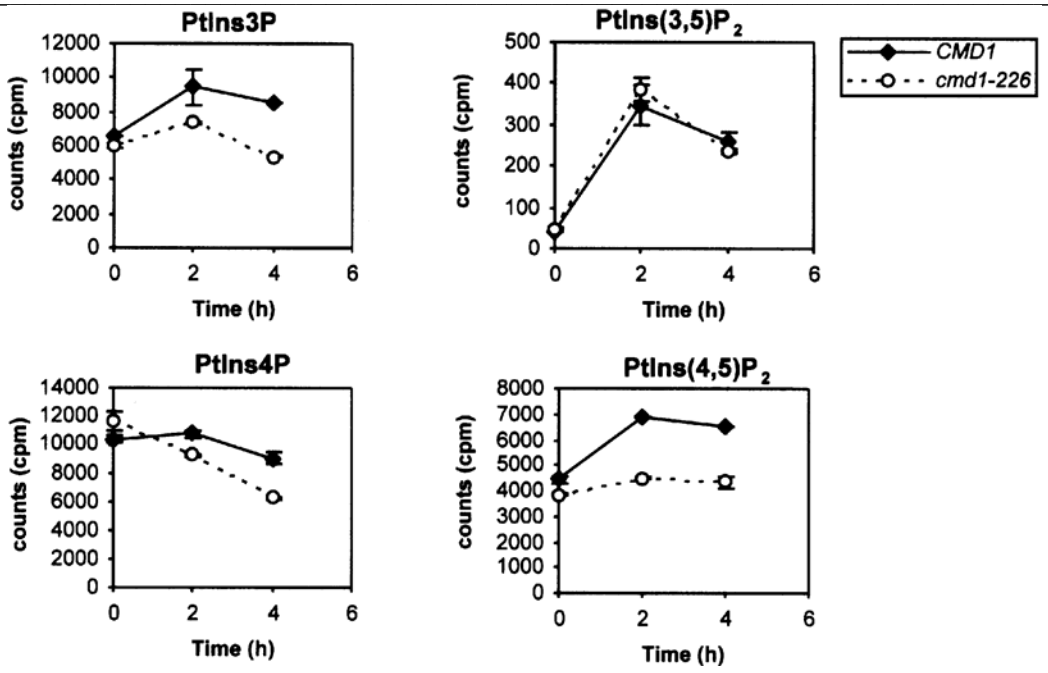

Figure 4 Calmodulin regulates PtdIns(4,5)P_2 levels *in vivo*

Wild-type (*CMD1*; DBY7462) and *cmd1-226* (DBY7446) strains were labelled at 24°C with [^{3}H]inositol and transferred to 37°C. Aliquots were collected at the indicated times, and the [^{3}H]inositol-labelled lipids from cell extracts were deacylated and separated by HPLC. Radioactivity associated with each lipid was quantified and corrected to total levels of phosphatidylinositol found in the control samples. Total phosphatidylinositol levels (c.p.m.) were as follows: *CMD1*, 0h, 663 147; 2h, 693 733; 4h, 687 418; *cmd1-226*, 0h, 753 572; 2h, 902 835; 4h, 617 489. Data represent means±S.E.M of two independent experiments.

Calmodulin is required for synthesis of PtdIns(4,5)P_2 *in vivo*

To determine if calmodulin acts upstream of MSS4, we examined phosphoinositide levels in the *cmd1-226* mutant. Cells were labelled with [^{3}H]inositol at 24°C, shifted to 37°C for various times, and assayed for phosphorylated phosphatidylinositol levels (Figure 4). As observed previously (147), a temperature up-shift induced production of PtdIns(4,5)P_2 in a wild-type strain (*CMD1*), due to activation of MSS4. PtdIns(4,5)P_2 levels increased approx. 2-fold within 1h of the temperature shift and remained at this level for at least 2h. In contrast, *cmd1-226* cells failed to induce PtdIns(4,5)P_2 synthesis upon temperature up-shift, indicating that calmodulin regulates PtdIns(4,5)P_2 levels. The *cmd1-226* mutation had little to no effect on the production of PtdIns3*P*, PtdIns4*P* and PtdIns(3,5)P_2, indicating that calmodulin regulates specifically PtdIns(4,5)P_2 levels. These findings provide further evidence that calmodulin acts upstream of MSS4, in particular, as an activator of MSS4.

Calmodulin and MSS4 signal independently of the PKC1-activated mitogen-activated protein kinase (MAPK) cascade

Organization of the actin cytoskeleton and cell integrity, both of which are affected in *mss4* and *cmd1* mutants, are controlled in part by the PKC1-dependent MAPK cascade (76). The PKC1-dependent MAPK cascade comprises BCK1/SLK1 (MAPK kinase kinase; 'MAPKKK'), MKK1 and MKK2 (MAPK kinases; 'MAPKKs') and MPK1/SLT2 (MAPK) (262). To investigate if calmodulin and MSS4 signal to the actin cytoskeleton via activation of this pathway, we examined whether overexpression of PKC1 or components of the MAPK cascade restore growth in *cmd1-226* and *mss4^{ts}* mutants. As shown in Figure 3, the growth defect of the *cmd1-226* mutants is weakly suppressed by overexpression of PKC1. Overexpression of components upstream of PKC1, such as the guanine nucleotide exchange factor ROM2 or the small GTPase RHO2, suppressed very weakly, if at all, the *cmd1-226* mutation. Expression of a constitutively active allele of *BCK1* (*BCK1-20*) or overexpression of MKK1 or MPK1 failed to suppress *cmd1-226* (Figure 3). Overexpression of PKC1 or components of the MAPK cascade failed to suppress the *mss4^{ts}* mutation (results not shown). These results suggest that the growth defects of the *cmd1-226* and *mss4^{ts}* mutants are not due solely to a lack of activation of the MAPK pathway downstream of PKC1. To determine if calmodulin and MSS4 are required for the activation of the PKC1-dependent MAPK pathway, we assayed activation of MPK1 in *cmd1-226* and *mss4^{ts}* cells. Shifting wild-type cells to 39°C for 1h resulted in activation of the cascade, as determined by Western blotting using an antibody specific for the activated form of MPK1 (263-265) (results not shown). Elevated temperature induced activation of MPK1 in *mss4^{ts}* cells to a level similar to that observed in wild-type cells. The *cmd1-226* mutation resulted in an even stronger and longer activation of MPK1 (results not shown). These results indicate that calmodulin and MSS4 are not required for activation of the PKC1-dependent MAPK pathway. However, calmodulin and possibly MSS4 may still signal via PKC1, but independently of the MAPK cascade, as suggested by the suppression of *cmd1-226* by multicopy *PKC1*.

DISCUSSION

We have shown previously (147) that MSS4 is the only PtdIns(4)*P* 5-kinase in *S. cerevisiae*, and that it is required for proper organization of the actin cytoskeleton. In the present study, we provide evidence that calmodulin activates MSS4-mediated synthesis of PtdIns(4,5)P_2, and that it is via MSS4 that calmodulin controls the actin cytoskeleton. First, cells lacking functional calmodulin or MSS4 proteins have similar phenotypes. Secondly, overexpression of MSS4 restores both growth and actin organization in the *cmd1-226* mutant. Finally, PtdIns(4,5)P_2 levels are reduced in cells lacking calmodulin.

How does calmodulin activate MSS4? Two models could explain how calmodulin regulates PtdIns(4,5)P_2 synthesis. First, calmodulin could activate a PtdIns 4-kinase that would, in turn, yield more substrate for MSS4. Alternatively, calmodulin could activate MSS4 independently of a PtdIns 4-kinase. Several lines of evidence suggest that calmodulin does not activate a PtdIns 4-kinase. First, unlike PtdIns(4,5)P_2, total cellular levels of PtdIns4*P* do not increase upon shift of wild-type cells to 37°C. Secondly, a calmodulin deficiency prevents heat-induced PtdIns(4,5)P_2 accumulation, but does not cause a significant change in PtdIns4*P* levels. Finally, overexpression of either one of the two *S. cerevisiae* PtdIns 4-kinases, PIK1 and STT4, failed to suppress the growth defect of the *cmd1-226* mutant (results not shown). Thus calmodulin may activate MSS4 independently of the PtdIns 4-kinases. Frequenin, another member of the calmodulin superfamily, has been shown to bind and stimulate PIK1 (135). This suggests that activation of PtdIns-kinases by Ca^{2+}-binding proteins may be a common theme. However, we could not detect a direct interaction between calmodulin and MSS4 using the yeast two-hybrid system (results not shown), suggesting that calmodulin may not activate MSS4 directly.

How do calmodulin and MSS4 control the organization of the actin cytoskeleton? One possibility is that they regulate the interactions between actin and the actin-binding proteins. Cell motility and changes in morphology initiated by binding of extracellular ligands to transmembrane receptors, for example during the activation of platelets or neutrophils, require rearrangement of the actin cytoskeleton. This is generally achieved by a transient increase in cytoplasmic Ca^{2+} concentration and by synthesis of PtdIns(4,5)P_2. Ca^{2+} leads to solubilization of the cytoskeleton via activation of proteins that disrupt the

actin network and inhibition of actin cross-linking proteins. Conversely, PtdIns(4,5)P_2 promotes actin polymerization by inhibiting actin-filament severing, capping and monomer-sequestering proteins (for review see (266)). The opposite effects of Ca^{2+} and PtdIns(4,5)P_2 have led to a two-step model for the reorganization of the actin cytoskeleton during cell spreading or crawling (267). First, Ca^{2+} induces the release of actin filaments from the membrane and depolymerization of the cytoskeleton. The second phase, characterized by repolymerization of actin, is triggered by synthesis and rearrangement of PPI. Our previous observations in yeast cells undergoing thermal stress support this model (147,195). Rearrangement of the actin cytoskeleton in response to heat is biphasic. A rapid disappearance of actin cables and depolarization of the actin cytoskeleton is followed by an MSS4-dependent phase in which actin cables reappear and the cytoskeleton repolarizes. Our finding that calmodulin is required for heat-induced activation of MSS4 suggests that calmodulin is involved in the second phase of actin rearrangement and might act as a negative feedback regulator of Ca^{2+}-induced actin depolarization and as a switch between actin depolarization and repolarization.

The suppression, albeit weak, of *cmd1-226* by multicopy *PKC1* suggests that calmodulin and MSS4 may also signal to the actin cytoskeleton via PKC1. PKC1 is activated by the GTP-bound form of the GTPase RHO1 or its homologue RHO2 (181,220). Surprisingly, we found that overexpression of either RHO2 or its GDP/GTP exchange factor ROM2 did not suppress the *cmd1-226* mutation. This may suggest that there are different pools of PKC1 regulated by different mechanisms. This notion is consistent with the observations that PKC1 displays punctate cytoplasmic localization and is also found associated with the plasma membrane at the site of bud growth, and that shifting cells to 37°C induces nuclear translocation only of PKC1 fractions initially localized at the plasma membrane (results not shown). Thus MSS4 and RHO1/2 might activate different pools of PKC1. However, if MSS4 does signal to the actin cytoskeleton via PKC1, this signaling appears to be independent of the PKC1-effector MAPK cascade, as MPK1 activation is not affected in *cmd1* or *mss4* mutants. The existence of a calmodulin-activated PKC1-dependent pathway parallel to the MAPK cascade is consistent with an earlier suggestion that there is functional redundancy of the Ca^{2+}- and MPK1-regulated pathways (268). The Ca^{2+}-activated protein calcineurin and the PKC1-activated MAPK pathway act in concert to

regulate transcription of *FKS2*, which encodes a subunit of the 1,3-β-glucan synthase required for cell-wall synthesis (269,270). Calcineurin and MPK1 also act in synergy to control the onset of mitosis by activating the SWI1 transcription factor at the transcriptional and post-translational levels respectively (271). In addition, the phenotypes of *glc7* cells, carrying a mutation in the catalytic subunit of type 1 protein serine/threonine phosphatase, suggests a role for this phosphatase in maintenance of cell-wall integrity, possibly via a PKC1-related pathway (272).

We also found that cells lacking functional MSS4 are defective in endocytosis. This provides additional support for the idea that PtdIns(4,5)P_2 is required for endocytosis. An involvement of PtdIns(4,5)P_2 in endocytosis was suggested previously by the finding that a dominant-negative isoform of PtdIns(4)*P* 5-kinase Iβ inhibits endocytosis of human colony-stimulating factor 1 receptor (259). Furthermore, key components of the clathrin-mediated endocytic machinery are PtdIns(4,5)P_2-binding proteins (258,260). Deletion of members of the synaptojanin inositol 5-phosphatases family in yeast also leads to reduced endocytosis (64), suggesting a role for PtdIns(4,5)P_2 turnover in endocytosis. Targeted disruption of the *synaptojanin 1* gene in mice leads to death shortly after birth (70). Neurons of mutant animals have elevated levels of PtdIns(4,5)P_2 and accumulate clathrin-coated vesicles at the synaptic vesicle cluster, the site where clathrin-mediated internalization of the synaptic vesicle membrane occurs. Interestingly, cells lacking calmodulin, like *mss4*^{ts} mutants, are defective in endocytosis (250,251). It remains to be determined if calmodulin also regulates endocytosis via activation of MSS4.

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3. MSS4 regulation and PI(4,5)P_2 effector in *Saccharomyces cerevisiae*

PI(4,5)P_2 synthesis and turnover are well studied processes and are important in several cellular processes such as the organization of the actin cytoskeleton. In mammalian cells many PIP5K isoforms exist and they are suggested to be regulated by the RHO GTPase family and phosphatidic acid (see 1.3.1). In *Saccharomyces cerevisiae* there is only one PIP5K, MSS4, (see 1.4.1.2 Fig 5) but the regulation is not thoroughly characterized. We wanted to understand in more detail how MSS4 is regulated and to identify and characterize possible interaction partner/s. In addition, we have studied possible effector/s of PI(4,5)P_2 which could link MSS4 function to the signaling cascade of TOR2 and the cell wall integrity pathway.

3.1 MSS4 can be part of a complex

Mammalian PIP5K interact with different proteins but the interaction modules have not been described. Upon heat shock, a shift from 24°C to 37°C, PI(4,5)P_2 levels increase dramatically (147). This suggests that the PI(4,5)P_2 synthesis might be regulated. MSS4 does not contain any known protein interaction module. Therefore we undertook the study if MSS4 could be part of a complex by using gel filtration chromatography. We observed that MSS4 eluted as a high molecular weight fractions of approximately 1000 kDa indicating the possibility that it could be part of a complex (Fig 3.1A). Detergent was added to these experiments to avoid aggregations that could be caused by lipid interaction. To investigate if this observation could be the result of dimerization of MSS4 co-immunoprecipitation experiments was carried out. A strain with MSS4 genomically tagged with TAP module was transformed with a plasmid containing HA-tagged MSS4. The co-immunoprecipitation experiment revealed no interaction between these two differentially tagged MSS4 (Fig 3.1B). Subsequently, we performed high scale purification to identify putative interaction partners. MSS4 tagged at the N-terminal with 3XHA was used for purification with cation exchange chromatography (which had been proven to give highly purified protein) and in parallel N-terminal (tandem affinity purification) TAP-tagged

MSS4 was purified. These purifications were repeated several times but no interaction partner was identified (data not shown). These results indicate that MSS4 is might be part of a complex and the component/s of the complex remains to be characterized.

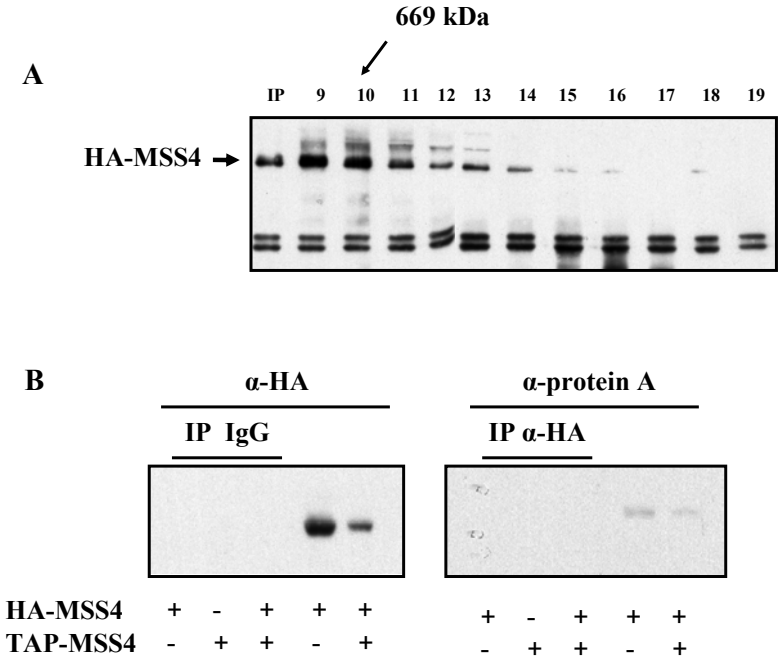

Figure 3.1 **MSS4 is part of a complex.**

(**A**) Gel filtration chromatography using a strain expressing HA-MSS4 (146). Each fraction immunoprecipitated with HA-antibody, visualized with HA antibody (see Experimental procedures). (**B**) 3XHA-MSS4 (146) expressed in strain containing genomic TAP-MSS4

3.2 Effectors of PI(4,5)P_2

MSS4 was previously shown to suppress the growth defect upon loss of TOR2 function (179). To understand the link between MSS4 in TOR2 signaling pathway and/or the cell wall integrity pathway we were interested in identifying the effectors of PI(4,5)P_2-signaling. For this study we focused first on characterization of known PH domain containing proteins. One of the putative PI(4,5)P_2-targets is the GEF of RHO1 ROM2 which is downstream of WSC1 and TOR2 (209). Since actin cytoskeleton is depolarized in a conditional mutant of *mss4* we used CAP2 as an indicator of the actin distribution. CAP2

localizes to the growing ends of actin filaments. ROM2 was tagged with yellow fluorescence protein (YFP) to compare simultaneously the localization of CAP2-CFP (cyano fluorescence protein). ROM2-YFP localizes to the bud tip and to the bud neck of small budded cells (Steffen Fredersdorf,unpublished results and Fig 3.2). To test if the levels of PI(4,5)P_2 are important for ROM2 localization it was observed in a *MSS4* mutant. Whereas in wild type, ROM2 localized to the bud tip and bud neck we observed a cytosolic signal of ROM2 upon loss of MSS4 function (Fig 3.2). This result suggests that PI(4,5)P_2 is important for proper localization of ROM2 and a link between MSS4 function and TORC2 signaling pathway

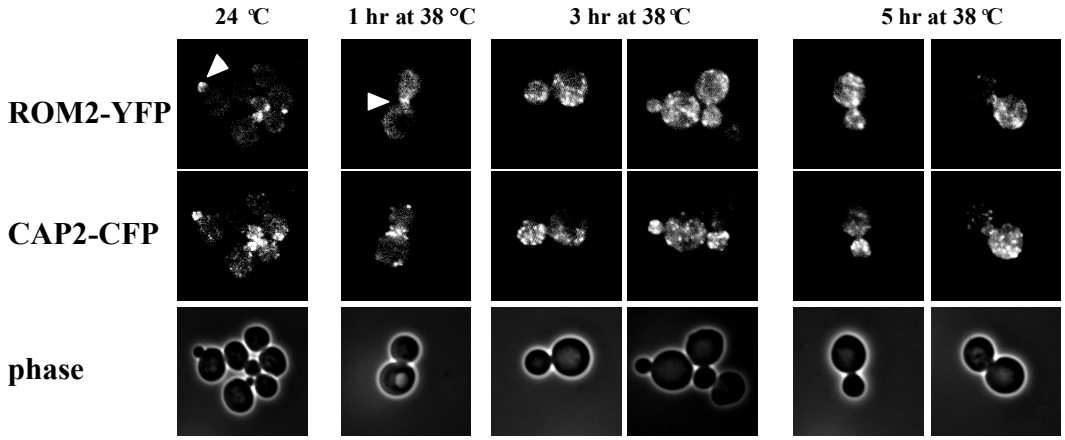

Figure 3.2 **ROM2 localization is dependent on PI(4,5)P_2 synthesis.**

Wild type cells (TB50) and *mss4^{ts}* were grown at 24°C, shifted to 37°C at the indicated time and processed for detection of ROM2-YFP and CAP2-CFP (see Experimental procedures).

Experimental Procedures

Strains, media

The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. All strains were isogenic derivatives of TB50. Rich media (YPD) and SD medium was as described previously (273). For tagging of MSS4 a PCR-fragment containing TAP was introduced at the N-terminus between promoter and START as described elsewhere (274). Yeast transformation was performed by the lithium acetate procedure (275). Correct integration was verified by PCR.

Gel filtration chromatography

Cells expressing a tagged version of MSS4 were grown in 500 ml of YPD to A_{600} of 0.8 at 30°C and harvested by centrifugation, and washed with cold water before resuspension in lysis buffer. Lysis buffer used to prepare cell extracts was PBS , 5% glycerol, 0.5% Tween 20 and phosphatase inhibitors (10 mM NaF, 10 mM NaN_3, 10 mM p-nitrophenylphosphate, 10 mM sodiumpyrophosphate, 10 mM ß-glycerophosphate and 1 mM phenylmethylsulfonyl fluoride) and protease inhibitor cocktail tablets (Roche). Cell lysate was obtained by glass bead lysis. Cell lysates were cleared with a 5 min, 500Xg spin, and the supernatant was passed through a 0.22 µm filter. Five milligrams of protein (in a volume of less than 0.5 ml) was loaded onto a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech). The flow rate was adjusted to 0.2 ml/minute, and fractions of 1 ml were collected. Elution profiles were analyzed by immunoprecipitation (1 hr incubation) and immunoblot and compared to the elution profile of known standards.

Immunoprecipitations

Yeast extracts from 500 ml culture grown in YPD to A_{600} of 0.8 at 30°C expressing tagged proteins of interest were prepared. An aliquot of extract containing 10 mg of protein was adjusted to 1 ml with lysis buffer plus inhibitors. For immunoprecipitations, 20 µl anti-HA (12CA5) cross-linked to Protein A-sepharose beads or 20 µl IgG Sepharose 6 fast flow (Amersham Pharmacia Biotech) were added and mixed for 4 hrs at 4°C. Beads were collected by centrifugation, washed extensively, and resuspended in 5X SDS-PAGE sample buffer for electrophoresis. After SDS-PAGE, proteins were blotted onto nitrocellulose

membranes, blocked in 5% dry milk powder in 1X PBS and 0.1% NP40, and incubated with primary antibody anti-HA (12CA5) or anti-protein A 1:10000 in blocking solution). Subsequently tagged proteins were detected using horseradish peroxidase-conjugated goat anti-mouse secondary antibodies and ECL reagents (Amersham Pharmacia Biotech).

Fluorescence microscopy

Cells of various strains were grown at 24°C in YPD. At A_{600} of 0.2, cells were shifted to 37°C for the time indicated. 1 ml aliquots were collected, cells were spun down and the pellet resuspended in SD medium to decrease background signal. YFP and CFP signal was visualized using fluorescence microscopy.

TABLE I

STRAIN	GENOTYPE
TB50	*MAT**a** leu2-3,112 uta3-52 trp1 his3 rme1 HML**a***
HM16-1C	*TB50 a mss4::HIS/* **pRS314*::3XHA-MSS4 URA3***
HM31-1C	*TB50**a** TAP-MSS4*
HM20-1C	*TB50a **mss4::HIS3MX6*/YCplac111::*MSS4**/ ROM2-YFP HIS /CAP2-CFP HIS*
HM21-3B	*TB50a **mss4::HIS3MX6*/YCplac111::*mss4-2^{ts}*** */ROM2-YFP HIS/CAP2-CFP HIS*
HM29-3D	*TB50a ROM2-YFP HIS/CAP2-CFP HIS*

4. Negative regulation of PI(4,5)P_2 levels by the INP51-associated proteins TAX4 and IRS4

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Abstract

Phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) is an important second messenger in signaling pathways in organisms ranging from yeast to mammals, but the regulation of $PI(4,5)P_2$ levels remains unclear. Here we present evidence that $PI(4,5)P_2$ levels in *Saccharomyces cerevisiae* are downregulated by the homologous and functionally redundant proteins TAX4 and IRS4. The EPS15 homology (EH) domain containing proteins TAX4 and IRS4 bind and activate the $PI(4,5)P_2$ 5-phosphatase INP51 via an NPF motif in INP51. Furthermore, the INP51-TAX4/IRS4 complex negatively regulates the cell integrity pathway. Thus, TAX4 and IRS4 are novel regulators of $PI(4,5)P_2$ and $PI(4,5)P_2$-dependent signaling. Furthermore, the interaction between TAX4/IRS4 and INP51 is analogous to the association of EPS15 with the 5-phosphatase synaptojanin in mammalian cells, suggesting that EPS15 is an activator of synaptojanin.

Introduction

Phosphoinositides are conserved from yeast to mammals as second messengers. They mediate signal transduction involved in many different cellular processes and thereby comprise a complex signaling system (6 45). One of the more thoroughly studied phosphoinositides is phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$). $PI(4,5)P_2$ was originally shown to be cleaved by phospholipase C to generate the two second messengers inositol-1,4,5- phosphate (IP_3) and diacylglycerol (DAG) (233). More recently, uncleaved $PI(4,5)P_2$ has also been shown to act as a second messenger (150,276). Uncleaved $PI(4,5)P_2$ acts by binding conserved domains in target proteins, such as the pleckstrin homology (PH) domain (10). In mammalian cells, $PI(4,5)P_2$ signaling is important in regulating vesicular transport, the organization of the actin cytoskeleton, and regulation of ion channels (19,67,277). In the model organism *Saccharomyces cerevisiae*, $PI(4,5)P_2$ is also essential and is produced by MSS4, the sole phosphatidylinositol 4-phosphate 5-kinase (PI4P 5-kinase) in yeast (147) (146).

One target of $PI(4,5)P_2$ signaling in *Saccharomyces cerevisiae* is the GDP/GTP exchange factor (GEF) ROM2. ROM2 contains a pleckstrin homology (PH) domain that binds $PI(4,5)P_2$ in the plasma membrane thus mediating the localization of ROM2 {Ozaki, 1996 #103} (132). ROM2 has two homologs, ROM1 and TUS1, that also harbour a

putative PH-domain {Ozaki, 1996 #103}(210). ROM2 and its homologs are components of the cell integrity pathway necessary for the cellular response to cell wall damage induced by stress such as heat shock (204,210). The response to cell wall damage includes a reorganization of the actin cytoskeleton and an upregulation of cell wall synthesis (183,191,195,278). Activation of the cell integrity pathway is mediated by its most upstream component, the cell wall sensor WSC1, that signals to ROM2 (265,279). Subsequently, ROM2 stimulates the exchange of GDP to GTP in the Rho GTPase RHO1, thereby activating RHO1 {Ozaki, 1996 #103}. The GTPase-activating protein (GAP) SAC7 converts RHO1-GTP into the inactive GDP-bound form (209). RHO1 in its active (GTP-bound) form has several effectors such as the glucan synthase FKS1 and the PKC1-MAP kinase cascade (280) (181,214,220). Activation of the MAP kinase MPK1 induces transcription of genes involved in cell wall biosynthesis, such as *CHS3* encoding chitin synthase III important for cell wall repair (191,192,278).

The ROM2-MAP kinase-signaling pathway is also an effector branch of the TOR signaling network. The TOR signaling network contains two structurally and functionally distinct complexes, TOR complex 1 (TORC1) and 2 (TORC2). TORC2 regulates the polarization of the actin cytoskeleton, and this regulation is via the ROM2-MAP kinase pathway (179,232). Interestingly, the PI4P 5-kinse MSS4 is required for the polarization of the actin cytoskeleton, and overexpression of MSS4 restores growth in conditional TORC2 mutants (147,179) (146,232). However, the link between PI(4,5)P_2 and TORC2 in the regulation of ROM2 and ultimately the actin cytosekeleton is not understood.

The phosphoinositide 5-phosphatases INP51, INP52 and INP53 mediate the turnover of PI(4,5)P_2 and are implicated in several cellular processes such as cell wall biosynthesis and the organization of the actin cytoskeleton (153,168). Heat shock has been observed to induce an increase in PI(4,5)P_2 levels, suggesting the existence of a mechanism regulating the levels of this phosphoinositide (147). However, the nature of this regulatory mechanism and, more specifically, the possible regulation of the phosphoinositide 5-phosphatases are not well understood. In mammalian cells, the INP family orthologue synaptojanin 1, interacts with several proteins involved in endocytosis in nerve terminals (66,71,74). To investigate the regulation of PI(4,5)P_2 turnover in *Saccharomyces cerevisiae*, we focused on INP51, INP52 and INP53 and their role in PI(4,5)P_2 signaling linked to the cell integrity pathway and the TORC2 pathway.

Here we present evidence for two novel positive regulators of the PI(4,5)P_2 5-phosphatase INP51, the redundant EPS15 homology (EH) domain-containing proteins TAX4 and IRS4. INP51 associates with TAX4 or IRS4 to form two complexes important for the turnover of PI(4,5)P_2 linked to the cell integrity pathway.

Experimental Procedures

Strains and media

The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. All strains were isogenic derivatives of TB50. Rich medium (YPD) and SD medium were as described previously (253). PCR cassettes were used to generate gene deletions and for tagging with HA, myc or TAP, as described (Wach 1994) (281)(Puig 2001). Yeast transformation was performed by the lithium acetate procedure (275). Correct integration was verified by PCR and tagged proteins were tested for their functionality.

Spot assay

Logarithmically growing cells were harvested and resuspended in 10 mM TRIS pH 7.4. The resuspended cells were diluted in a tenfold dilution series. 3 µl of each dilution (10x, 100x, 1,000x and 10,000x diluted) were spotted on an YPD plate. Growth was scored after two days at 30°C or 37°C.

INP51 purification

Cells expressing a tagged or untagged (mock purification) version of INP51 were grown in five litres of YPD to A_{600} of 0.8 at 30°C and harvested by centrifugation, and washed with cold water before resuspension in lysis buffer. Lysis buffer used to prepare cell extracts was PBS, 5% glycerol. 0.5% Tween 20 and phosphatase inhibitors (10 mM NaF, 10 mM NaN_3, 10 mM p-nitrophenylphosphate, 10 mM sodiumpyrophosphate, 10 mM ß-glycerophosphate 1 mM phenylmethylsulfonyl fluoride) and protease inhibitor cocktail tablets (Roche). Cell lysate was obtained by glass bead lysis. Cell lysates containing approximately 700 mg protein were cleared with a 5 min, 500Xg spin, diluted with lysis buffer to 10 mg/ml, and subsequently passed over an ion exchange resin (SP-sepharose). The resin was washed twice with lysis buffer and twice with lysis buffer containing 50 mM potassium acetate. Bound proteins were eluted with lysis buffer containing 600 mM potassium acetate. The eluate was precleared over a Protein A-Sepharose (Amersham Pharmacia Biotech) column prior to the addition of anti-HA (12CA5) cross-linked to Protein A-sepharose beads. After 5X washing with lysis buffer, immunoprecipitated

proteins were visualized by silver staining (282). Analysis of protein bands by mass spectrometry was performed as described (232).

Immunoprecipitation

Yeast extracts from cells (500 ml culture in YPD grown to A_{600} of 0.8 at 30°C) expressing tagged proteins of interest were prepared. An aliquot of extract containing 10 mg of protein was adjusted to 1 ml with lysis buffer plus inhibitors. For immunoprecipitations, 20 □l anti-HA (12CA5) or anti-myc (9E10) cross-linked to Protein A-sepharose beads were added and mixed for 4 hrs at 4°C. Beads were collected by centrifugation, washed five times with 1 ml lysis buffer, and resuspended in 5X SDS-PAGE sample buffer for electrophoresis. After SDS-PAGE proteins were blotted onto nitrocellulose membranes, blocked in 5% dry milk powder in 1 X PBS and 0.1% NP40, and incubated with primary antibody anti-HA (clone 12CA5) or anti-myc (clone 9E10) 1:10000 in blocking solution). Subsequently tagged proteins were detected using horseradish peroxidase-conjugated goat anti-mouse secondary antibodies and ECL reagents (Amersham Pharmacia Biotech).

Phosphoinositide analysis

Strains were grown at 30°C to A_{600} of 0.7 in medium containing 50 µCi/ml [^{3}H]Inositol (Amersham). The cells were harvested by centrifugation and resuspended in one-fifth of the original volume. Aliquots (200 µl) were collected, and the cells were arrested by the addition of 500 µl of methanol. After drying samples in vacuo, lipid extraction and analysis were performed as described previously (127).

Differential centrifugation

Differential centrifugation was performed as described previously (283).

Indirect immunofluorescence

Logarithmically growing cells containing HA-INP51, myc-TAX4 or myc-IRS4 were fixed for 2 h in the growth medium supplemented with formaldehyde (3.7% final) and potassium phosphate buffer (100 mM final, pH 6.5). Cells were washed and resuspended in sorbitol buffer (1.2 M sorbitol and 100 mM potassium phosphate, pH 6.5). Cell walls were digested

for 45 min at 37°C in sorbitol buffer supplemented with ß-mercaptoethanol (20 mM final) and zymolyase 20T (12.5 mg/ml; Seigagaku Corporation). Spheroblasts were fixed on poly-L-lysine–coated glass slides and permeabilized with PBT (53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl, 1% BSA, and 0.1% Triton X-100). Immunofluorescence directed against the HA-epitope was performed by application of a primary antibody anti-HA (clone 12CA5) or anti-myc (clone 9E10) at a dilution of 1:1,000 in PBT for 2 h, and subsequently of a Cy3-conjugated rabbit anti–mouse IgG (Molecular Probes), diluted 1:1,000 in PBT, for 90 min. Washed cells were examined with a Zeiss Axiophot microscope (100X objective). Immunofluorescent detection of FKS1 was performed as described previously (195).

MAP kinase activation assay

YPD cultures of logarithmically growing cells at 24°C were harvested and cell extracts were prepared as described previously (263). Protein concentrations of extracts were determined by using Bradford Assay (BIO-RAD). Samples were denatured by addition of 5X SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer and heating at 95°C for 5 min. A total of 25 □g of protein (for MPK1 protein detection) or 40 □g of protein (for phosphorylated MAPK detection) was loaded for standard SDS-PAGE (10% acrylamide) and Western blot (Ausubel et al 1998). For immunodetection, a goat anti-MPK1 antibody (clone yN-19, 1:1000 dilution; Santa Cruz) and rabbit anti-phospho-p44/42 MAP kinase (THR202/Tyr204) antibody (1:1000; Cell Signaling) were used. The anti-MPK1 and anti-phospho MAPK were verified to specifically recognize activated MPK1 under heat stress conditions (data not shown). Secondary antibodies were horseradish peroxidase-conjugated anti-goat (anti-MPK1) or anti-rabbit (anti-phospho MAPK) secondary antibody and detection by ECL reagents (Amersham Pharmacia Biotech).

Actin staining

Logarithmically growing cells were fixed in formaldehyde (3.7%) and potassium phosphate buffer (100 mM, pH 6.5), and stained with TRITC-phalloidin (Sigma) to visualize actin, as described previously (257).

Chitin staining

1 ml of cells at A_{600} of 0.5 were collected by centrifugation and washed with water. Cells were then incubated in a solution with 0.5 mg/ml calcofluor white (CFW; Sigma) for 5 min to visualize chitin. To remove residual CFW, cells were washed twice with water. Cells were examined with a Zeiss Axiophot microscope (100X objective).

Results

Disruption of *INP51* restores growth of a *tor2* mutant

To understand further how turnover of $PI(4,5)P_2$ is important for $PI(4,5)P_2$ signaling. We tested if the phosphoinositide 5-phosphatase INP51, INP52 or INP53 antagonizes MSS4 signaling. We asked if the lack of any *INP* gene mimics MSS4 overexpression in suppressing a TOR2 signaling defect. A deletion of each *INP* gene was introduced into a temperature-sensitive *tor2* (*tor2*^{ts}) mutant defective in the organization of the actin cytoskeleton. The double mutants were grown at permissive (30°C) and restrictive temperatures (37°C) to determine if *INP* mutations can suppress the *tor2*^{ts} mutation. Deletion of only *INP51*, *INP52* or *INP53* conferred no growth defect at any temperature (data not shown). Interestingly, deletion of *INP51*, but not deletion of *INP52* or *INP53*, suppressed the growth defect of *tor2*^{ts} cells. The *inp51 tor2*^{ts} cells grew almost as well as wild type cells and as well as *tor2*^{ts} cells overexpressing MSS4 (Fig. 1 and data not shown). This suggests that INP51, but not INP52 or INP53, antagonizes the role of MSS4 in the TORC2 signaling pathway. Curiously, although *inp51* suppressed the growth defect of a *tor2*^{ts}, it did not appear to suppress the actin defect of the *tor2*^{ts} mutant (data not shown).

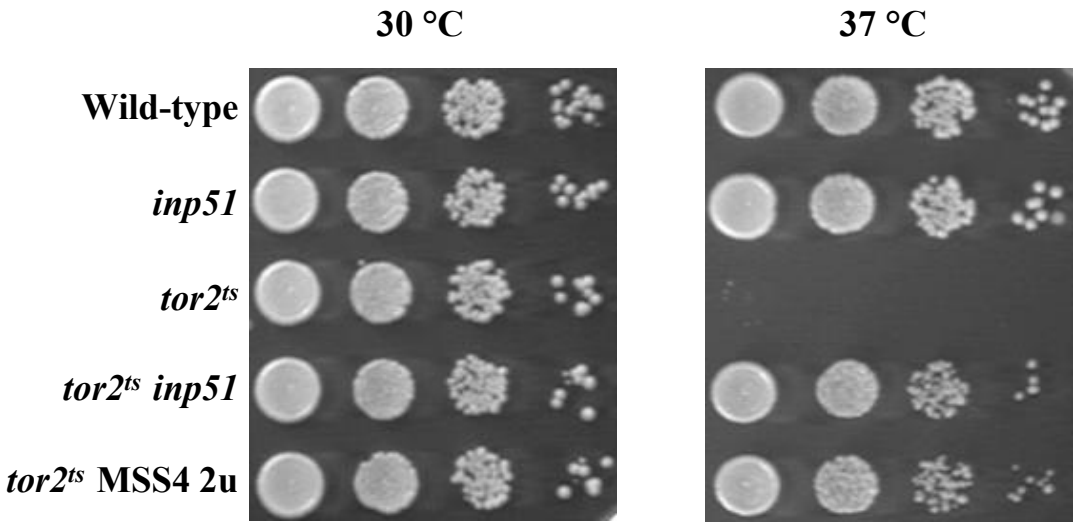

Figure 1. **Disruption of *INP51* restores growth of a *tor2* conditional mutant.**
Cells from wild-type (TB50**a**), *inp51, tor2*^{ts}, *tor2*^{ts} *inp51* and *tor2*^{ts} transformed with pMSS4 were serially diluted in YPD liquid medium, spotted onto YPD medium and incubated at 30 °C or 37 °C. *tor2*^{ts} cells are not viable at 37 °C.

***INP51* synthetically interacts with mutations affecting the cell integrity pathway**

The finding that an *inp51* deletion suppresses the growth defect of a *tor2*^{ts} mutant is similar to the previous observation that activation of the cell integrity pathway suppresses the growth defect of a *tor2*^{ts} mutant (204), and suggests that INP51 may antagonize the cell integrity pathway. Thus, we investigated if an *inp51* mutation interacts with mutations affecting components of the cell integrity pathway. Specifically, we asked if an *inp51* deletion suppresses or enhances the effect of mutations in *WSC1, ROM2, SAC7* or *MPK1*. We observed that deletion of *INP51* suppresses the growth defects of *wsc1* and *rom2* cells at 37°C and 30°C, the restrictive temperatures of these mutants, respectively (Fig. 2A). Conversely, the combination of *inp51* with *sac7* conferred a synthetic growth defect (Fig. 2C). Finally, the growth defect of an *mpk1* mutant grown at restrictive temperature (38°C)

was not suppressed by *INP51* deletion (data not shown). The above results show that *INP51* genetically interacts with at least some components of the cell integrity pathway. Deletion of *INP51* suppresses mutations in positive components of the pathway (*WSC1* and *ROM2*) and causes a synthetic defect when combined with a mutation in a negative element of the pathway (*SAC7*). The nature of these interactions suggests that the phosphoinositide phosphatase INP51 negatively regulates signaling through the cell integrity pathway. This conclusion is consistent with the previous conclusions (120,179,232) (132) that the phosphoinositide kinase MSS4 and PI(4,5)P_2 act positively on the TORC2 and cell integrity pathways.

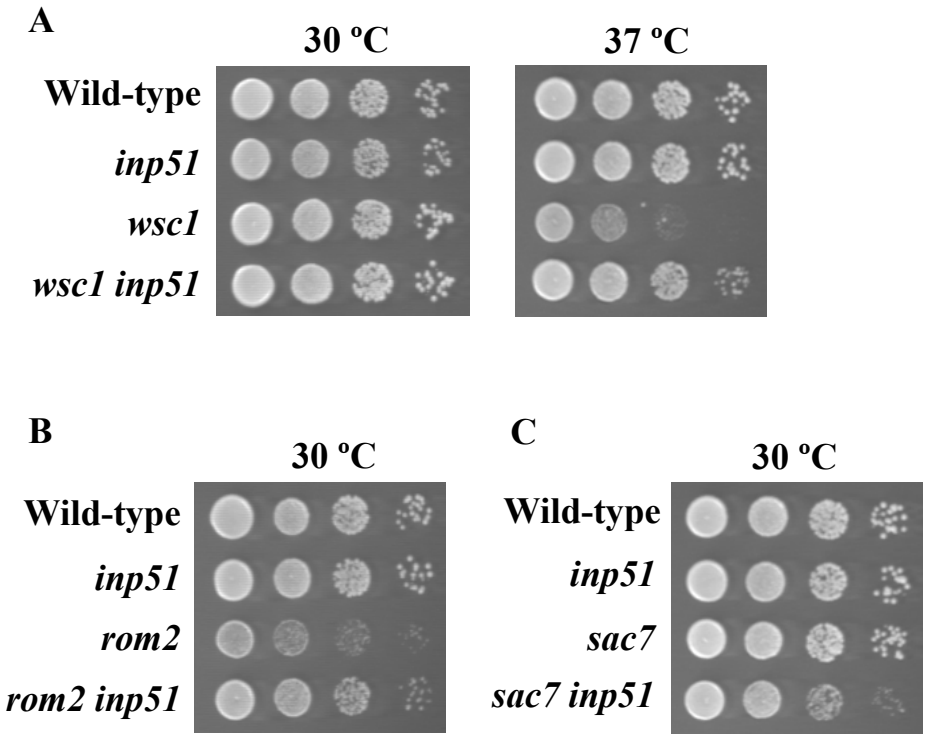

Figure 2. ***INP51*** **synthetically interacts with components of the cell wall integrity pathway.**

(A) Wild-type, *inp51*, *wsc1* and *wsc1 inp51* cells were serially diluted in YPD liquid medium, spotted onto YPD medium and grown at 30 °C or 37 °C. *wsc1* cells have a growth defect at 37 °C. (B) Wild-type, *inp51*, *rom2* and *rom2 inp51* cells were grown on YPD medium at 30°C. *rom2* cells have a growth defect at 30 °C. (C) Wild-type, *inp51*, *sac7* and *sac7 inp51* cells were grown on YPD at 30 °C.

INP51 associates with the EH domain-containing proteins TAX4 and IRS4

In mammalian cells, the kinases and phosphatases that determine PI(4,5)P_2 levels are regulated by interacting proteins (69,71,245,284-286). Indication that the level of PI(4,5)P_2 is regulated in *Saccharomyces cerevisiae* has been suggested previously by the observation that PI(4,5)P_2 levels increase upon heat shock (147). However, proteins regulating such kinases and phosphatases in *Saccharomyces cerevisiae* remain to be identified. To identify potential INP51 regulatory proteins, we opted to isolate INP51 interacting proteins using a biochemical approach described previously (232). A functional, epitope-tagged version of INP51 (INP51-HA) was constructed and purified from yeast cell extracts as described in Experimental Procedures. Two homologous proteins co-purified with INP51, IRS4 and an uncharacterized protein encoded by the open reading frame YJL083w (Fig. 3) which we named TAX4. The interaction between INP51 and TAX4 or IRS4 was confirmed by co-immunoprecipitation using epitope-tagged versions of TAX4 (TAX4-myc) and IRS4 (IRS4-myc), as described in Experimental Procedures (Fig. 4). We did not observe interaction between TAX4 and IRS4 by co-immunoprecipitation of heterologously tagged versions (TAX4-TAP and IRS4-myc) of these proteins (data not shown). IRS4 was previously identified in a screen for mutants involved in rDNA silencing as a mutant with increased rDNA silencing (287). Untagged TAX4 has an apparent molecular weight of 77 kDa and a predicted size of 68.7 kDa. Untagged IRS4 is observed as two bands between 70 and 75 kDa, slightly larger than the predicted size of 68.8 kDa. TAX4 and IRS4 have an overall identity of 31% and contain a C-terminal EPS15 homology (EH)-domain. The EH domains of TAX4 and IRS4 are 64% identical. The EH domain, conserved from yeast to human, is a protein-protein interaction domain of approximately 100 amino acids that interacts specifically with short motifs containing an asparagine-proline-phenylalanine (NPF) core (288,289). INP51 has a C-terminal NPF motif (290) which is important for the interaction between INP51and TAX4 or IRS4. The absence of the NPF motif in INP51 abolishes the interaction between INP51 and TAX4 or IRS4, as assayed by co-immunoprecipitation (data not shown). Our results shows that INP51, via its NPF-motif, interacts separately with the two EH domain-containing proteins TAX4 and IRS4, suggesting that INP51 forms separate INP51-TAX4 and INP51-IRS4 complexes.

There are additional EH-containing proteins in *Saccharomyces cerevisiae*, including PAN1, END3 and EDE1 (257,291,292). Interestingly, a conditional *pan1* mutation shows synthetic interaction with an *inp51* mutation (158). Since PAN1 and END3 belong to the same protein complex, we tested if PAN1 and/or END3 interact with INP51. We could not detect, by co-immunoprecipitation, an interaction between INP51 and PAN1 or END3, suggesting that INP51 interacts specifically with TAX4 and IRS4.

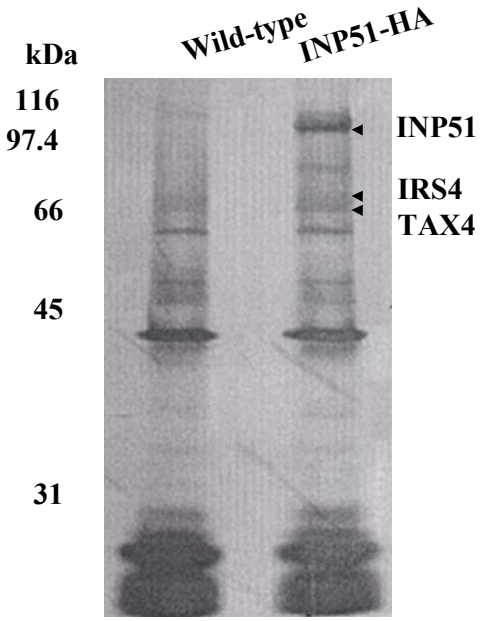

Figure 3. **INP51 copurifies with two proteins, TAX4 and IRS4**.

Silver stained gel of INP51-HA purification (see Experimental Procedures) from TB50 cells (wild-type) and HM47-1C cells (INP51-HA). Protein bands unique to the INP51-HA purification are indicated. These bands were excised and identified by mass spectrometry (see Experimental Procedures).

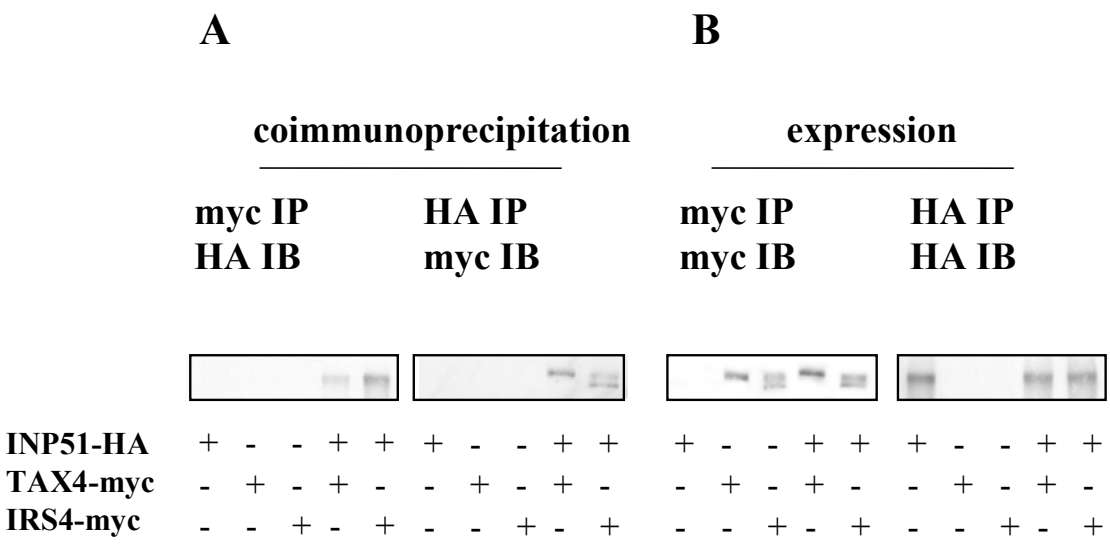

Figure 4. INP51 co-immunoprecipitates with TAX4 and IRS4.

(A) Lysates from cells expressing INP51-HA (HM47-1C), TAX4-myc (HM92-), IRS4-myc (HM104-2D) or co-expressing INP51-HA and TAX4-myc (HM96-1B), or INP51-HA and IRS4-myc (HM119-2B) were subjected to coimmunoprecipitation by immunoprecipitation (IP) with anti-myc and immunoblot (IB) with anti-HA or IP with anti-HA and IB with anti-myc. (B) For expression controls each strain in (A) was subjected to anti-myc IP and IB with anti-myc or IP with anti-HA and probed with anti-HA.

TAX4 and IRS4 positively regulate INP51

The interaction of INP51 with TAX4 and IRS4 led us to examine a possible involvement of TAX4 and IRS4 in the function of INP51. *TAX4* and *IRS4* are non-essential genes, as shown previously (293). Furthermore, cells lacking both *TAX4* and *IRS4* (a *tax4 irs4* double mutant) did not confer a detectable growth defect on standard rich or minimal media at various temperatures (Fig. 6 and data not shown).

To determine the function of TAX4 and IRS4, we measured the levels of all detectable *S. cerevisiae* phosphoinositides, PI(3)P, PI(4)P, PI(3,5)P_2 and PI(4,5)P_2, in *inp51*, *tax4*, *irs4* and *tax4 irs4* mutant cells. The double mutant *tax4 irs4* was examined

anticipating the likelihood that the structurally homologous TAX4 and IRS4 proteins are also functionally homologous. We observed that an *inp51* mutant has higher levels of $PI(4,5)P_2$ than wild type, as observed previously (156), but is not altered in the levels of other phosphoinositides (Fig. 5). The deletion of *TAX4* or *IRS4* did not affect the level of any phosphoinositide. However, the double deletion mutant *tax4 irs4*, like the *inp51* mutant, exhibited a significant and specific increase in the level of $PI(4,5)P_2$. Our data suggest that TAX4 and IRS4 indeed act redundantly, as positive regulators of INP51 activity and thereby $PI(4,5)P_2$ turnover.

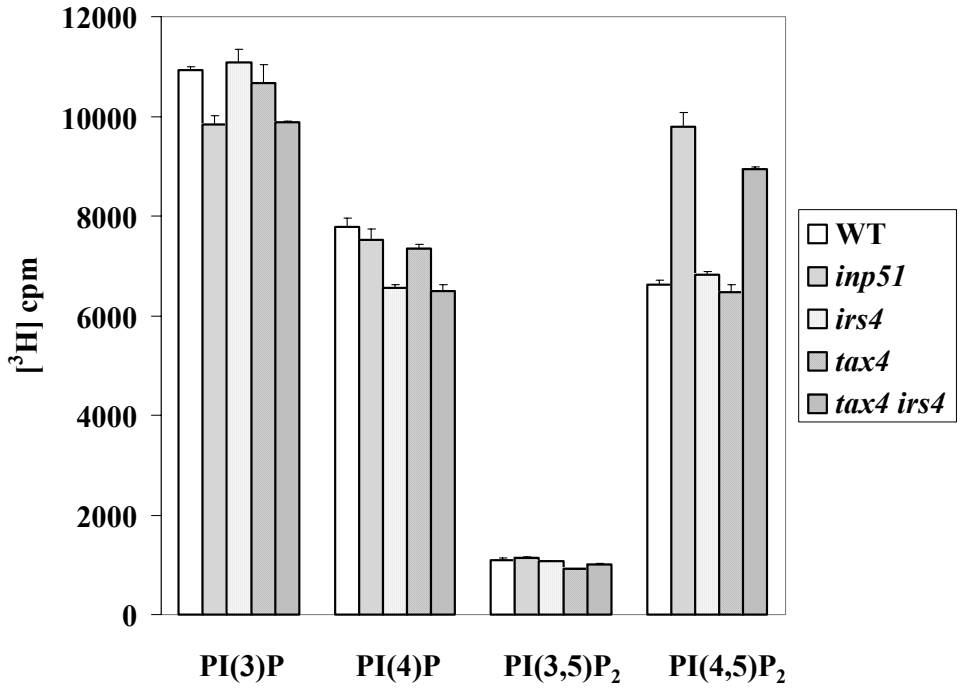

Figure 5 **$PI(4,5)P_2$ level is increased in an *inp51* mutant and in a *tax4 irs4* mutant.**
Wild-type (WT), *inp51*, *irs4*, *tax4* and *tax4 irs4* cells were labeled at 30°C with [3H]inositol, and [3H]inositol–containing lipids from the cell extracts were deacylated, separated by HPLC, and quantified.

Since TAX4 or IRS4 appears to be required for INP51 activity, we asked if the *tax4 irs4* double mutation, like *inp51*, genetically interacts with *TOR2* and components of the cell integrity pathway. We combined the *tax4 irs4* double mutation with $tor2^{ts}$, *rom2*, or

sac7. In agreement with the positive role of TAX4 and IRS4 on INP51 activity, *tax4 irs4* suppressed the growth defect of a *tor2*^{ts} mutant at non-permissive temperature (Fig. 1 and 6), to the same extent as deletion of *INP51* or overexpressed MSS4. The growth defect of a *rom2* mutant was also suppressed by *tax4 irs4* mutation. Finally, combination of *tax4 irs4* with a *sac7* mutation produced a synthetic growth defect similar to the combination of *inp51* and *sac7* (Fig. 2 and 7). Deletion of *TAX4* or *IRS4* alone showed no synthetic interaction with components of the cell integrity pathway (data not shown). Our results suggest that TAX4 or IRS4, like INP51, is important for PI(4,5)P_2 turnover linked to TORC2 signaling and the cell integrity pathway.

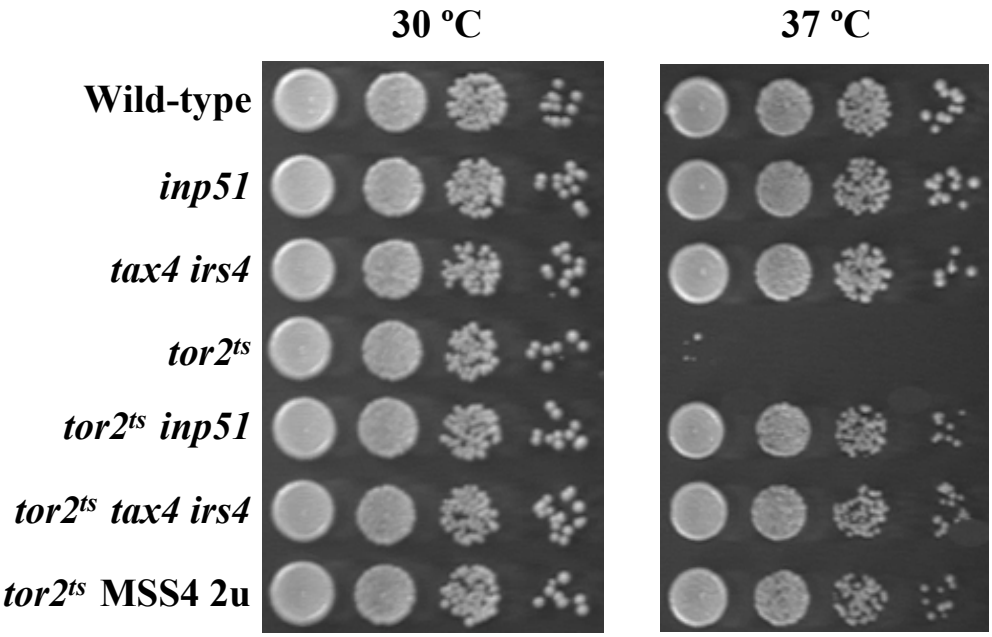

Figure 6. **Deletion of *TAX4* and *IRS4* restores growth of a *tor2* conditional mutant.**
Cells from wild-type, *inp51*, *tax4 irs4*, *tor2*^{ts}, *tor2*^{ts} *inp51, tor2*^{ts} *tax4 irs4* and *tor2*^{ts} transformed with pMSS4, were serially diluted in YPD liquid medium, spotted onto YPD medium and grown at 30 °C or 37 °C. *tor2*^{ts} cells are not viable at 37 °C.

To investigate the mechanism by which TAX4 and IRS4 regulate INP51, we examined the cellular localization of TAX4, IRS4 and INP51, by indirect immunofluorescence on strains expressing epitope-tagged versions of these proteins. We observed an intracellular punctate staining pattern for both TAX4 and IRS4. However, for unknown reasons, we were unable to detect a signal for INP51, and were thus unable to determine by this assay if TAX4 or IRS4 is required for the correct localization of INP51. By differential centrifugation (see Experimental Procedures), we observed that INP51 is a cytosolic protein and this localization did not change in a *tax4 irs4* double mutant, suggesting that TAX4 or IRS4 does not regulate the subcellular localization of INP51.

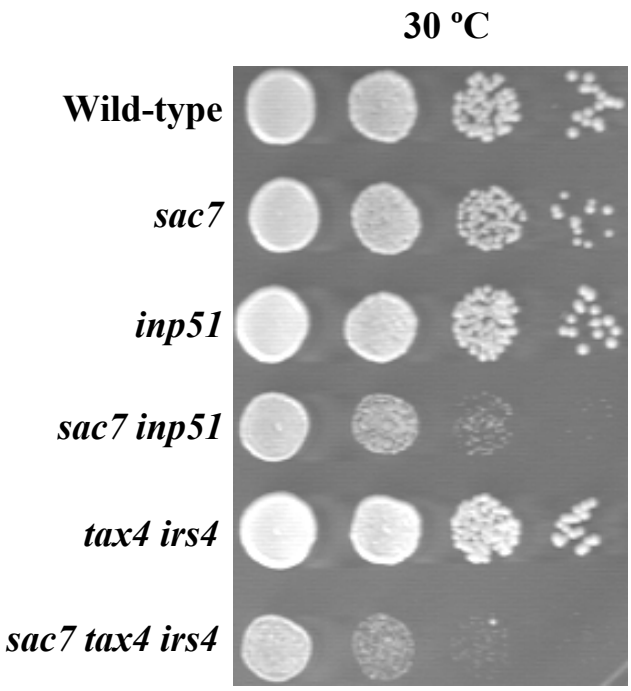

Figure 7 **Null mutation of both *TAX4* and *IRS4* interacts synthetically with *SAC7* deletion mutant.**

Wild-type, *sac7, inp51*, *sac7 inp51, tax4 irs4* and *sac7 tax4 irs4* were serially diluted in YPD liquid medium, spotted onto YPD medium and grown at 30 °C.

INP51, TAX4 and IRS4 negatively regulate MPK1

The genetic interactions described above suggest that INP51, TAX4 and IRS4 negatively regulate signaling through the cell integrity pathway. However, the above experiments (and data not shown for TAX4 and IRS4) did not reveal a genetic interaction between MPK1 and the INP51 complexes. To investigate further a possible functional interaction between MPK1 and the INP51 complexes, we examined MPK1 phosphorylation in wild type cells and in mutant cells containing *rom2*, *sac7*, *inp51*, *tax4*, *irs4*, *tax4 irs4,* or any possible combination of *rom2* or *sac7* with *inp51, tax4, irs4,* or *tax4 irs4*. Upon stimulation of the cell integrity pathway MPK1 is phosphorylated and thereby activated (222). MPK1 phosphorylation was analyzed with an antiphospho p44/p42 MAPK antibody that also specifically recognizes the phosphorylated form of MPK1. The amount of total MPK1 was assayed with a separate antibody that recognizes all forms of MPK1. As shown previously, MPK1 phosphorylation is almost undetectable in wild type cells grown at 23°C (non-inducing condition) but increases upon shift to 39°C (222). We examined the phosphorylation state of MPK1 in wild type cells and mutant cells grown at low temperature (24°C) and at high temperature (39°C). The level of MPK1 phosphorylation in heat shocked cells (shift to 39°C for 30 minutes and 60 minutes) was not altered in any of the mutants examined (data not shown). However, the *sac7*, *sac7 inp51, sac7 tax4 irs4* mutations affected the basal level (24°C) of MPK1, as follows. A *sac7* mutation resulted in a slight increase in MPK1 phosphorylation but also a similar increase in the amount MPK1 protein, compared to wild type (Fig. 8). The *sac7 inp51* and *sac7 tax4 irs4* cells exhibited a 10 fold increase in MPK1 phosphorylation and a 2.5 fold increase in MPK1 protein level compared to the *sac7* cells. Thus, *inp51* and *tax4 irs4* mutations cause constitutive MPK1 activity, although this effect is detectable only when *inp51* and *tax4 irs4* are combined with a *sac7* mutation. Furthermore, as suggested by some of the genetic interactions described above, these findings confirm that INP51 complexes indeed negatively regulate signaling through MPK1 and the cell integrity pathway.

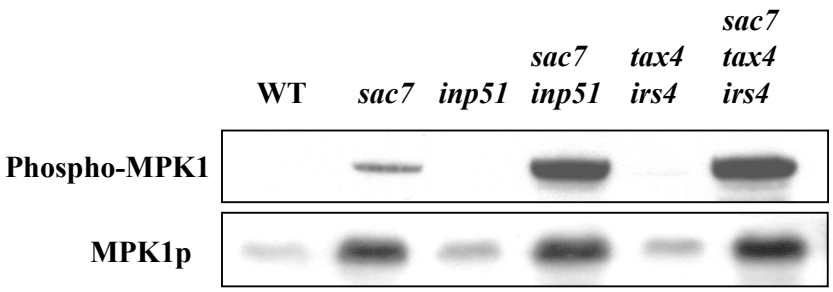

Figure 8. **MPK1 phosphorylation and protein level increases in *inp51 sac7* deletion mutant and *tax4 irs4 sac7* deletion mutant.**

MPK1 activation and protein levels were analyzed in WT, *sac7*, *inp51*, *sac7 inp51*, *tax4 irs4* and *sac7 tax4 irs4* at 25 °C. Analysis of (first panel) activated MPK1 with antibodies recognizing specific Thr/Tyr phosphorylation, and (second panel) MPK1 protein level with antibodies recognizing MPK1.

The distribution of chitin is negatively regulated by INP51, TAX4 and IRS4

To further investigate the regulation of MPK1 signaling by the INP51 complexes, we examined MPK1 readouts in *sac7 inp51* and *sac7 tax4 irs4* cells. In particular, we examined the actin cytoskeleton, glucan synthase (FKS1) and chitin (see Introduction) in *sac7*, *inp51*, *tax4 irs4*, *sac7 inp51* and *sac7 tax4 irs4* cells grown at low (24°C) and high temperature (39°C). Heat stress transiently depolarizes both the actin cytoskeleton and the glucan synthase (195). The above mutant cells behaved like wild type cells with regard to the actin cytoskeleton and FKS1 (data not shown), suggesting that INP51, TAX4 and IRS4 do not regulate signaling of the cell integrity pathway to these targets. However, the amount and distribution of chitin was changed in some mutants. In wild type cells, chitin is most visible at the neck and at a future bud site (Fig. 9). In the *sac7* and *tax4 irs4* cells chitin distribution was slightly depolarized while in *sac7 inp51* and *sac7 tax4 irs4* cells we observed a striking increase in chitin signal and depolarization of chitin distribution. In

sac7 inp51 and *sac7 tax4 irs4* cells, the chitin was clearly distributed over the entire cell surface in unbudded cells, to the mother cell surface in small budded cells, and to both mother and daughter cell surfaces in large budded cells (Fig. 9). The change in chitin amounts and distribution suggests that INP51, TAX4 and IRS4 regulate a specific branch of the cell integrity pathway affecting chitin.

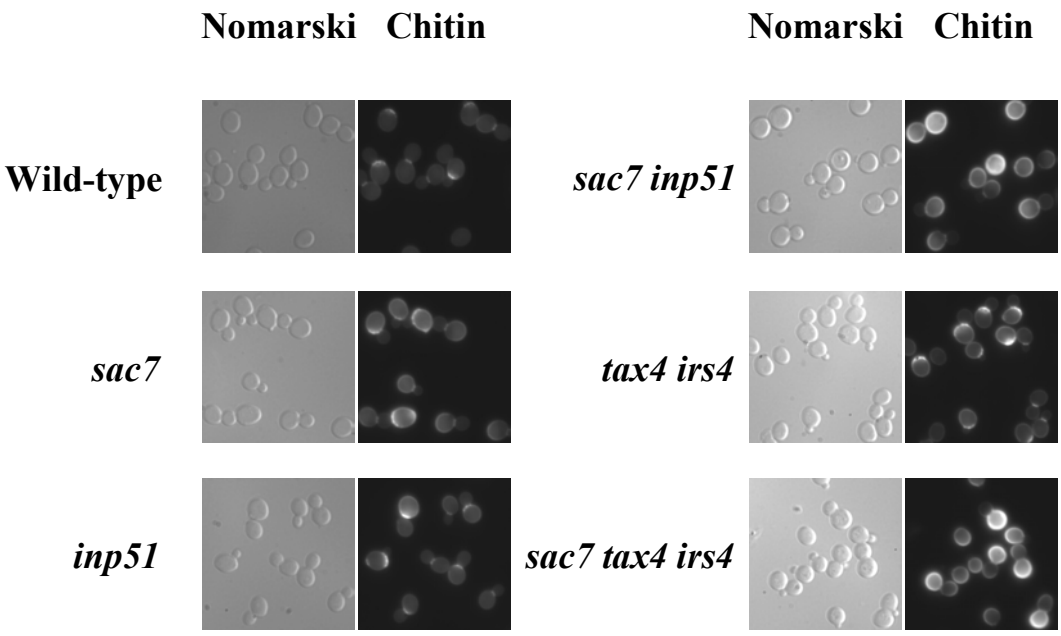

Figure 9 **Chitin deposition is delocalized in *sac7 inp51 deletion mutant* and in *sac7 tax4 irs4* deletion mutant.**

Cells from logarithmically grown cultures (25 °C) were fixed with formaldehyde, and stained with calcuofluorwhite. The cells were examined with Nomarski optics and by fluorescence to visualize the chitin.

To investigate if the affect on chitin is the cause of the observed growth defect of *sac7 inp51* and *sac7 tax4 irs4* cells (Fig. 7), *CHS3* was deleted in these mutants. *CHS3* encodes chitin synthase III that is responsible for 90% of the chitin produced in the cell, although deletion of *CHS3* does not cause a growth defect (294-296). A deletion of *CHS3* in *sac7 inp51* and *sac7 tax4 irs4* mutants reduced the amount of chitin to wild type levels but did not suppress the growth defect (data not shown). This suggests that targets of the

cell integrity pathway other than chitin could be affected (hyperactivated) and causing the growth defect observed in *sac7 inp51* and *sac7 tax4 irs4* cells.

Discussion

Here we present two proteins, TAX4 and IRS4, that are novel partners of the phosphoinositide 5-phosphatase INP51. The structurally homologous and functionally redundant TAX4 and IRS4 proteins positively regulate INP51 activity, and thereby negatively regulate PI(4,5)P_2 levels. Furthermore, the INP51 complexes (INP51-TAX4 and INP51-IRS4) negatively regulate signaling through the cell integrity pathway including the MAP kinase MPK1.

TAX4 and IRS4 contain an EPS15 homology (EH) domain. EH domains are found mainly in proteins involved in endocytosis, organization of the actin cytoskeleton, or signal transduction, and interact specifically with short motifs containing an asparagine-proline-phenylalanine (NPF) core. INP51 contains an NPF motif (290) which is important for the interaction with TAX4 or IRS4, and which is not found in any other phosphoinositide phosphatase in *Saccharomyces cerevisiae*. Thus, the interaction between INP51 and TAX4/IRS4 is similar to the interaction between the mammalian phosphoinositide 5-phosphatase synaptojanin (NPF-containing) and EPS15, suggesting a conserved regulatory mechanism(71). The functional consequence of EPS15 binding to synaptojannin is not known, but our findings suggest that this binding may result in activation of synaptojanin phosphatase activity.

How does TAX4 or IRS4 activate INP51? The EH domain has been suggested to play an important role in recruiting proteins to a specific cellular location(297). Our studies did not reveal a change in INP51 localization in the absence of TAX4 and IRS4, suggesting that TAX4 and IRS4 regulate INP51 by a mechanism other than recruitment. Furthermore, we did not detect a change in the binding of TAX4 or IRS4 to INP51 in response to several different types of cell wall stress, including heat shock and detergent treatment (data not shown). Future studies will be required to determine the mechanism by which TAX4 or IRS4 activates INP51.

We also present evidence that INP51, TAX4 and IRS4 genetically interact with two signaling pathways, the TORC2 signaling pathway and the cell integrity pathway. The mechanism by which *inp51* and *tax4 irs4* mutations suppress the growth defect of a *tor2*

mutant remains unclear, but could be through the activation of the cell integrity pathway. The *inp51* or *tax4 irs4* mutations activate MPK1, and the cell integrity pathway is a downstream effector pathway of TORC2. The activation of MPK1, which is detected only when *inp51* or *tax4 irs4* is combined with a *sac7* mutation, could be a result of increased RHO1 activity (balance toward the active, GTP-bound form of RHO1). The *sac7* mutation eliminates a RHO1 GAP and thereby prevents down regulation of RHO1, whereas *inp51* or *tax4 irs4* causes an increase in the level of $PI(4,5)P_2$ which could then activate RHO1 via the PH domain in the RHO1 GEFs.

The combined action of a GAP (SAC7) and a phosphoinositide phosphatase (INP51) could be a conserved mechanism to achieve proper spatial and temporal regulation of a signaling pathway, in this particular case, signaling through the cell integrity pathway. The GAP and phosphoinositide phosphatase combination has been observed in other organisms such as *Dictyostelium discoideum*, where the phosphoinositide phosphatase Dd5P4 contains both a $PI(4,5)P_2$ 5-phosphatase catalytic domain and a GAP catalytic domain (20). This domain combination is also present in the human $PI(4,5)P_2$ 5-phosphatase OCRL1 that when mutated causes mental retardation and kidney and eye failure (oculocerebrorenal Lowe syndrome) (60,63,298).

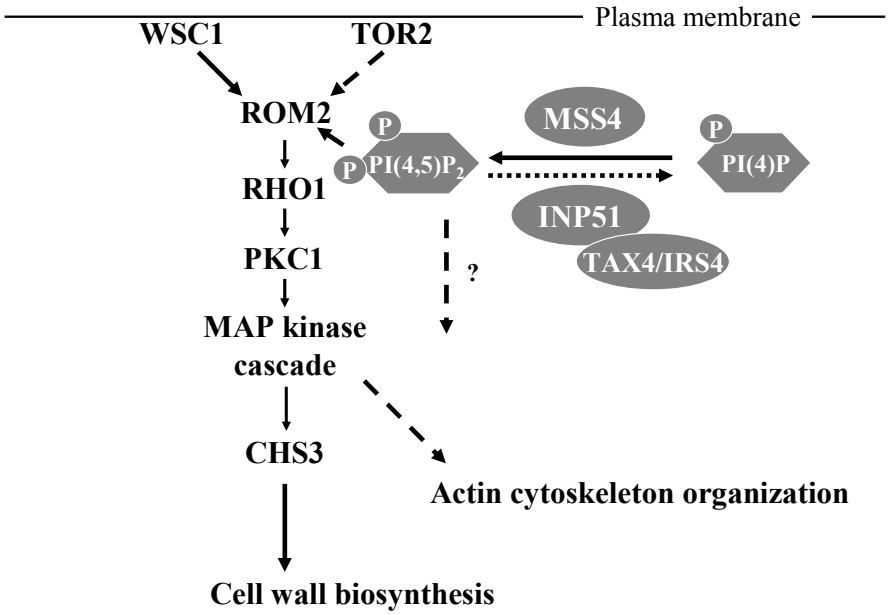

Figure 10. **Model showing INP51 complex regulating PI(4,5)P_2 signaling linked to the cell wall integrity pathway.**

PI(4,5)P_2 synthesis and turnover is regulated by MSS4 and INP51 respectively. INP51 activity is controlled by TAX4 and IRS4. PI(4,5)P_2 signalling impinges on PKC1 pathway controlled by two independent pathways, the TORC2 signaling pathway and cell wall integrity pathway. PI(4,5)P_2 could signal to ROM2 or through an unknown parallel pathway.

5. CONCLUSION AND PERSPECTIVES

Phosphorylated phosphoinositides (PPI) have been implicated in many cellular processes. Their essentiality has been shown by the implications of defective phosphoinositide metabolism in human disorders (34). Despite the identification and characterization of many phosphoinositide kinases and phosphatases their regulation mechanisms are almost unknown. However, the knowledge is increasing as exemplified by PI(3,5)P_2 biosynthesis and signaling (124,127,128). Further understanding how and when phosphoinositide kinases and phosphatases are regulated is therefore of great importance.

Here we present that the activity of the PIP5K of *Saccharomyces cerevisiae*, MSS4, is regulated by calmodulin, CMD1, but the direct activating mechanism has not been elucidated. Calmodulin is involved in many different cellular processes and the role of CMD1 and MSS4 signaling is most probably the organization of the actin cytoskeleton and endocytosis (249). The effectors that link CMD1 and MSS4 signaling are not known but there are several putative targets binding PI(4,5)P_2 and involved in the organization of the actin cytoskeleton such as actin-regulating proteins and GEFs (see 1.4.3). Another possibility is the signaling to components of the cell wall integrity pathway and the TOR2 signaling pathway since we observed link to PKC1. These two pathways have in common the PH domain containing effector protein ROM2. PI(4,5)P_2 is localized to the entire cell periphery as visualized by the GFP-tagged PH domain of PLCγ (167). However, ROM2 is localized only to the bud tip in small budded cells or to the bud-neck in larger budded cells. We observed the importance of PI(4,5)P_2 levels for ROM2 localization. This localization was also observed by (132) and is important for MSS4 signaling. This indicates that the localization is mediated through lipid interaction but additional membrane interaction such as protein-protein interaction is probably needed. Under active growing conditions PI(4,5)P_2 could play a role in the recruitment of proteins to the plasma membrane together with protein-protein interaction as could be the case with the PI(4,5)P_2-binding GEF ROM2. However, the overexpression of this specific GEF is not able to suppress the growth defect upon loss of CMD1 function suggesting another link between CMD1 and MSS4.

To understand more about the complex $PI(4,5)P_2$-signaling identification and characterization of proteins binding $PI(4,5)P_2$ is needed. More and more proteins are being identified revealing that not only the PH domain is important but also the ENTH domain. In addition, there is no clear consensus sequence for the PH domain making it difficult to identify all proteins containing PH domain and if the function of binding $PI(4,5)P_2$ is conserved. Interestingly, not all PH domains bind $PI(4,5)P_2$ suggesting the necessity to define the three dimensional domain structure necessary for lipid binding as a tool for the identification of proteins to find the exact $PI(4,5)P_2$-binding module. $PI(4,5)P_2$ has been shown to only be present at the plasma membrane implicating that PH-containing proteins localized at or near the plasma membrane are more likely the targets of this particular lipid. Identification of additional $PI(4,5)P_2$-binding proteins requires perhaps purification using $PI(4,5)P_2$ as a bait. This approach has been attempted by (299) identifying ARAP3 where $PI(3,4,5)P_3$ was covalently linked to beads. This method would also allow the identification of not yet defined lipid-binding domains. This approach has been attempted several times but identifying very few proteins even if there are over 100 of proteins suggested to interact with PPI. We also attempted to identify $PI(4,5)P_2$-binding proteins by using $PI(4,5)P_2$-coupled beads but no interaction candidates were identified. One reason could be the transient interaction with the PPI.

$PI(4,5)P_2$ has been shown to be both a regulator but also the precursor of messenger (IP_3 and DAG) making it difficult to distinguish between these two branches (7). Nevertheless, the existence of domains specifically binding $PI(4,5)P_2$ affirm the importance of this molecule. On the other hand it is intriguing that in *Saccharomyces cerevisiae* that overexpression of *MSS4* rescues the loss of the unique function of TOR2 (the organization of the actin cytoskeleton) while overexpression of *PLC1* is able to rescue both the unique and the shared function (nutrient sensing) (179). PLC1 and MSS4 have very different functions. Whereas MSS4 is essential for proper organization of the actin cytoskeleton and endocytosis PLC1 has been implicated in completely different processes like Ca^{2+} signaling and nutritional and stress-related responses (146,147,173,177,184,300). It will be of great interest to define the separate function of $PI(4,5)P_2$ on the one hand and IP_3 and DAG on the other hand in *Saccharomyces cerevisiae* to elucidate the distinct functions in the TORC2 signaling pathway.

The regulation mechanism of MSS4 remains unclear although there is evidence that MSS4 performs a cytoplasm-nuclear shuttling (152). It has been suggested that casein kinase I phosphorylates MSS4 which has also been observed in *Schizosaccharomyces pombe* (152,301). However, the significance of MSS4 phosphorylation in PI(4,5)P_2-signaling remains unclear. In mammalian cells, PIP5K have been shown to interact with RHO, RAC and ARF isoforms but this has yet not been observed in yeast. The identification of interactors might be dependent on specific conditions. Indication that PI(4,5)P_2 levels are regulated has been suggested previously by the fact that PI(4,5)P_2-levels increase upon heat shock (147).

The existence of specific PPI phosphatases suggest that these lipids need to be regulated as exemplified in mammalian cells. The PI(4,5)P_2 5-phosphatase synaptojanin 1 interacts with several proteins involved in the organization of the actin cytoskeleton and endocytosis such as amphiphysin (66). There are several candidates for interaction with the synaptojanin-like proteins in *Saccharomyces cerevisiae* such as the orthologue of amphiphysin RVS167 but interactions has not been observed (302). Here we present the synaptojanin-like INP51 as a specific PI(4,5)P_2 5-phosphatase that requires the presence of two EH domain containing proteins, TAX4 and IRS4 for its proper function. Our study suggests that the INP51 complex might interact via the EH-domain of TAX4 and IRS4, with the NPF-motif of INP51 (data not shown). The EH-NPF interaction is a conserved mechanism and in mammalian cells it is an important step in the targeting of proteins to specific cellular compartments (297). In *Saccharomyces cerevisiae* the EH-containing proteins with known functions such as PAN1, END3 and EDE1 are involved in endocytosis and partly in the organization of the actin cytoskeleton (196,291,292). In mammalian cells, the majority of the characterized EH-containing proteins such as EPS15 (interacts with synaptojanin 1), POB1 and intersectin 1 and 2 are involved in endocytosis and the organization of the actin cytoskeleton (303-305). Besides the EH-NPF interaction being conserved our finding that the interaction component is PI(4,5)P_2 phosphatase suggests a further common feature.

In addition, we show that INP51 together with TAX4 and IRS4 negatively regulates the cell wall integrity pathway and the TORC2 signaling pathway. This is opposed to MSS4 function and in agreement to our model that MSS4 is responsible for the production of PI(4,5)P_2 and INP51 for the turnover of PI(4,5)P_2 (see model Fig 4.9). The synthetic interaction observed in the *sac7 inp51* mutant strongly implicates a dual negative regulation

of the cell wall integrity pathway. This suggest that INP51 together with SAC7 is needed to control the cell wall integrity pathway to prevent excess cell wall synthesis which is observed in *sac7 inp51* double mutants. Interestingly, Dd5P4 is an inositol 5-phosphatase in *Dictyostelium discoideum* that contains a GAP domain important for its function (20). This domain combination is also present in humans, represented by the OCRL1 enzyme, suggesting that this mechanism, combination of GAP and 5-phosphatase activity, is conserved (60). This two way-mechanism of regulation could have evolved to prevent signaling when not needed.

In general, studies focusing in understanding $PI(4,5)P_2$-signaling and regulation in mammalian cells could give insights how for example plasma membrane signaling pathways involved in the remodelling of the actin cytoskeleton could be used as a tool to inhibit uncontrolled migration of malignant tumor cells (18). More specifically to the model organism *Saccharomyces cerevisiae*, future studies will be required to determine upstream signals of the INP51 complex as well as the direct targets of $PI(4,5)P_2$-signaling. Increased knowledge in the construction mechanism of the fungal cell wall would also give insights in further development of new antifungal compounds (190).

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Ich erkläre hiermit, dass ich diese Dissertation “Phosphatidylinositol 4,5-bisphosphate turnover by INP51 regulates the cell wall integrity pathway in Saccharomyces cerevisiae” nur mit der darin angebenen Hilfe verfasst und bei keiner anderen Fakultät eingericht habe.

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