Mammalian inositol polyphosphate 5-phosphatase II can compensate for the absence of all three yeast Sac1-like-domain-containing 5-phosphatases

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Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] plays a complex role in generating intracellular signalling molecules, and also in regulating actin-binding proteins, vesicular trafficking and vacuolar fusion. Four inositol polyphosphate 5-phosphatases (hereafter called 5-phosphatases) have been identified in *Saccharomyces cerevisiae*: Inp51p, Inp52p, Inp53p and Inp54p. Each enzyme contains a 5-phosphatase domain which hydrolyses PtdIns(4,5)P_2, forming PtdIns4*P*, while Inp52p and Inp53p also express a polyphosphoinositide phosphatase domain within the Sac1-like domain. Disruption of any two yeast 5-phosphatases containing a Sac1-like domain results in abnormalities in actin polymerization, plasma membrane, vacuolar morphology and bud-site selection. Triple null mutant 5-phosphatase strains are non-viable. To investigate the role of PtdIns(4,5)P_2 in mediating the phenotype of double and triple 5-phosphatase null mutant yeast, we determined whether a mammalian PtdIns(4,5)P_2 5-phosphatase, 5-phosphatase II, which lacks polyphosphoinositide phosphatase activity, could correct the phenotype of triple 5-phosphatase null mutant yeast and restore cellular PtdIns(4,5)P_2 levels to near basal values. Mammalian 5-phosphatase II expressed under an inducible promoter corrected the growth, cell wall, vacuolar and actin polymerization defects of the triple 5-phosphatase null mutant yeast strains. Cellular PtdIns(4,5)P_2 levels in various 5-phosphatase double null mutant strains demonstrated significant accumulation (4.5-, 3- and 2-fold for Δ*inp51*Δ*inp53*, Δ*inp51*Δ*inp52* and Δ*inp52*Δ*inp53* double null mutants respectively), which was corrected significantly following 5-phosphatase II expression. Collectively, these studies demonstrate the functional and cellular consequences of PtdIns(4,5)P_2 accumulation and the evolutionary conservation of function between mammalian and yeast PtdIns(4,5)P_2 5-phosphatases.

Key words: gene rescue, knockout, PtdIns(4,5)P_2.

INTRODUCTION

Phosphatidylinositols play complex signalling roles in both mammalian and yeast cells. The turnover of these ubiquitous minor membrane lipids following extracellular stimuli regulates cell growth and death [1], vesicular trafficking [2] and actin cytoskeletal rearrangement [3]. Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] serves as a precursor to both soluble inositol phosphates [4] and phosphorylated phosphatidylinositols [5], and, via interactions with actin-binding proteins, regulates actin cytoskeletal rearrangement [3]. Cellular levels of PtdIns(4,5)P_2 are maintained by its synthesis from phosphatidylinositol by specific lipid kinases [6,7], hydrolysis by phospholipase C following agonist stimulation [4], and dephosphorylation by specific lipid phosphatases designated inositol polyphosphate 5-phosphatases (hereafter called 5-phosphatases). This extensive enzyme family regulates PtdIns(4,5)P_2 concentrations by dephosphorylating the phosphate at the 5-position, forming phosphatidylinositol 4-phosphate (PtdIns4*P*) [8,9]. Nine mammalian 5-phosphatases have been characterized, while four isoforms have been identified in the yeast *Saccharomyces cerevisiae*.

Mammalian 5-phosphatases demonstrate distinct substrate specificities, dephosphorylating the 5-position phosphate from both phosphoinositides and inositol phosphates [10]. Recent studies of gene-targeted deletion of various 5-phosphatase isoforms in mice have demonstrated the significant role that these enzymes play in regulating cellular function. Mice lacking SHIP (SH2-containing inositol phosphatase), which principally hydrolyses PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4 and has a restricted expression to haematopoietic cells, demonstrate massive increases in white cells, which infiltrate the spleen and lungs [11–13]. Although humans with mutations in the Lowe's protein demonstrate Lowe's oculocerebrorenal syndrome, mental retardation, renal failure and cataracts [14], gene-targeted deletion of this 5-phosphatase in mice results in no phenotype [15]. Similarly, mice lacking 5-phosphatase II are normal, suggesting functional redundancy of these two enzymes. Gene-targeted deletion of 5-phosphatase II together with the Lowe's protein is lethal *in utero*. Mice lacking the neural synapse 5-phosphatase synaptojanin demonstrate neurological abnormalities and die shortly after birth [16]. Collectively, these studies demonstrate the important role of 5-phosphatase enzymes in regulating apoptosis, synaptic vesicle recycling, cell growth and development. However, as these 5-phosphatases each hydrolyse multiple phosphatidylinositol-derived messenger molecules, the precise mechanisms by which these cellular derangements occur are currently unclear.

Four 5-phosphatases have been identified and partially characterized in the yeast *S. cerevisiae* [17–20]. Three enzymes, designated Inp51p, Inp52p and Inp53p (also known as Sjl1p, Sjl2p and Sjl3p, for 'synaptojanin like') [21], contain an N-

Abbreviations used: 5-phosphatase, inositol polyphosphate 5-phosphatase; GroPtdIns, glycerophosphatidylinositol; Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II, Δ*inp51*Δ*inp52*Δ*inp53* triple mutant with 5-phosphatase II expression under control of the GAL1 promoter.

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terminal Sac1-like domain that demonstrates identity with yeast Sac1p, a central catalytic 5-phosphatase domain and C-terminal proline-rich sequences. The Sac1-like domains of mammalian synaptojanin and yeast Inp52p and Inp53p, but not Inp51p, possess intrinsic polyphosphoinositide phosphatase activity that hydrolyses PtdIns3*P*, PtdIns4*P* and PtdIns(3,5)P_2 to PtdIns [22]. A fourth yeast 5-phosphatase, Inp54p, has been identified which contains a 5-phosphatase domain but no other recognized signalling motifs [23]. The 5-phosphatase domain of all the yeast 5-phosphatases hydrolyses PtdIns(4,5)P_2, forming PtdIns4*P* [20]. Single null mutants of Δ*inp51*, Δ*inp52* and Δ*inp53* are viable [18]. However, single Δ*inp51* mutants display increases in cell growth at low temperatures. The double mutants Δ*inp51*Δ*inp52*, Δ*inp52*Δ*inp53* and Δ*inp51*Δ*inp53* demonstrate impaired cell growth [17], abnormalities in vacuole morphology, plasma membrane invaginations and cell wall thickening [19,19a,21]. Polarity of budding and actin abnormalities, as well as aberrant mitochondrial organization, have also been described [21]. Endocytic defects have been detected in Δ*inp51*Δ*inp52* null mutants and, to a lesser degree, Δ*inp52*Δ*inp53* double mutants [21]. In addition, Inp53p has been implicated in clathrin-mediated protein sorting at the *trans*-Golgi network [24]. Deletion of all three Sac1-like-domain-containing yeast 5-phosphatases is lethal.

The relative contribution of each yeast 5-phosphatase in the maintenance of phosphoinositide levels *in vivo* is complicated by the overlapping substrate specificity of each isoform, as all hydrolyse PtdIns(4,5)P_2 forming PtdIns4*P*. In addition, the Sac1-like domain of Inp52p and Inp53p expresses polyphosphoinositide phosphatase activity [22]. Analysis of cell lysates for PtdIns(4,5)P_2 5-phosphatase activity has revealed, in the single null mutants, a reduction in enzyme activity, which was most marked in the Δ*inp53* mutant [19,19a]; the double null mutants showed decreases in cellular PtdIns(4,5)P_2 5-phosphatase activity compared with the wild type (Δ*sjl1*Δ*sjl2*, 64 %; Δ*sjl1*Δ*sjl3*, 22 %; Δ*sjl2*Δ*sjl3*, 14 %). Analysis of the endogenous lipids by TLC, which does not distinguish between the phosphoinositide isomers PtdIns(4,5)P_2 and PtdIns(3,5)P_2, demonstrated a 2-fold increase in PtdInsP_2 in the Δ*inp51* single mutant and the Δ*inp51*Δ*inp52* and Δ*inp51*Δ*inp53* double null mutants, but not in the Δ*inp52*Δ*inp53* strain [17]. HPLC analysis of specific phosphoinositides has only been reported for the Δ*inp52*Δ*inp53* null mutant, and no accumulation of PtdIns(4,5)P_2, PtdIns3*P* or PtdIns4*P* was detected [22]. Therefore the phenotype, which is observed in all double null 5-phosphatase mutants, does not necessarily correlate with phosphoinositide levels.

In the present study, we demonstrate that the phenotype associated with deletion of the yeast Sac1-like-domain-containing 5-phosphatases correlates with a significant accumulation of PtdIns(4,5)P_2, and can be rescued by a mammalian isoform, 5-phosphatase II (also called the 75 kDa 5-phosphatase) [25]. This mammalian PtdIns(4,5)P_2 5-phosphatase does not contain a Sac1-like domain, indicating that the regulation of PtdIns(4,5)P_2 by the 5-phosphatase domain, rather than that of PtdIns, PtdIns3*P*, PtdIns4*P* or PtdIns(3,5)P_2 by the Sac1-like domain, contributes to the phenotype observed in double and triple yeast 5-phosphatase null mutant yeast strains.

MATERIALS AND METHODS

Materials

Restriction and modifying enzymes were obtained from Promega or New England Biolabs. Oligonucleotides were purchased from the Microbial Biotechnology and Diagnostic Unit, Monash University. *myo*-[^{3}H]Inositol, [α-^{32}P]dCTP and [γ-^{32}P]dATP were from NEN Life Science Products. Reagents for culture media were obtained from Oxoid (Basingstoke, U.K.), unless otherwise noted. Sequencing of all PCR products was performed using the ABI Prism BigDye Terminator Cycle Sequencing kit from Perkin Elmer Applied Biosystems (Foster City, CA, U.S.A.), and sample electrophoresis was performed at the Microbial Biotechnology and Diagnostic Unit, Monash University. All other reagents, unless otherwise stated, were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Genetic manipulations

The genes for *INP51*, *INP52* and *INP53* were disrupted with the *URA3*, *HIS3* and *TRP1* genes respectively. Specifically, an ~ 1.4 kb internal segment of *INP51* (472–1949 bp) was PCR-amplified from *S. cerevisiae* genomic DNA with added restriction sites (*Xba*I and *Hin*dIII), and cloned into pBluescript. The ~ 1.3 kb *URA3* gene was excised from plasmid pJJ242 by digestion with *Eco*RI and *Pvu*II, and was ligated in place of the 232 bp (nucleotides 1218–1449) *Eco*RI/*Eco*RV fragment in the *INP51* PCR construct. Diploid *S. cerevisiae* (W303) were transformed by electroporation with the entire cDNA fragment excised from pBluescript (*Not*I/*Xho*I), encoding *URA3* flanked by *INP51*. Ura+ transformants were screened for homologous insertion of the construct by PCR, using an internal *URA3* primer and the 3′-primer of the *INP51* construct.

Disruption of the *INP52* gene was performed using the *HIS3* gene. The *HIS3* gene was PCR-amplified from plasmid pRS303, and complementary flanking regions to *INP52* and pRS were added to produce a fragment encoding *HIS3* flanked by *INP52* coding sequences. Homologous recombination between the flanking *INP52* regions resulted in the insertion of the *HIS3* cassette and replacement of 1747–2721 bp in *INP52*. This construct was electroporated into diploid *S. cerevisiae* (W303) and His+ transformants were selected. Homologous insertion of the construct was determined by PCR, as for the Δ*inp51* transformants.

The disruption of the *INP53* gene was performed in an analogous manner to *INP52* disruption. *TRP1* was PCR-amplified from plasmid pRS304 with flanking regions from *INP53* corresponding to 1868–1912 bp and 2822–2866 bp. Homologous recombination between the flanking *INP53* regions resulted in insertion of the *TRP1* cassette and replacement of nucleotides 1913–2821 in *INP53*. This construct was electroporated into diploid *S. cerevisiae* (W303) and Trp+ transformants were selected. Homologous insertion of the construct was determined by PCR, as for the Δ*inp51* and Δ*inp52* transformants.

Following selection of diploid cells containing the relevant construct, diploid cells were induced to sporulate and tetrads were dissected to obtain haploid cells containing the disrupted genes. The single mutants were then mated and sporulated by standard techniques to obtain the haploid double null mutants Δ*inp51*Δ*inp52*, Δ*inp51*Δ*inp53* and Δ*inp52*Δ*inp53*. To obtain a triple mutant, the Δ*inp51* null mutant was mated with the Δ*inp52*Δ*inp53* mutant. Diploid cells containing all three mutant genes (Δ*inp51*Δ*inp52*Δ*inp53*) demonstrated normal growth and viability, but the haploid cells were non-viable.

5-Phosphatase II was PCR-amplified from cDNA encoding mouse 5-phosphatase II between nucleotides 108 and 3203. An alternatively spliced CAAX-less variant of 5-phosphatase II was used (GenBank accession number AY007563). The primers used were: 5-prime, ggactagtttgacagctcagcatgga (which encodes nucleotides 108–128 of 5-phosphatase II and a novel *Spe*I site); 3-prime, gggtcgacaattgtggctgccttaa (which encodes nucleotides 3184–3203 plus a novel *Sal*I site). The 3.1 kb PCR product was cloned into the *Spe*I and *Sal*I sites of the multicloning site of the

Table 1 Strains of *S. cerevisiae* used in the present study

With the exception of yeast strains W303 and W303α, which were generously provided by Dr D. Germain (Peter MacCallum Institute, Melbourne, Australia), the strains listed were developed in the present study (see the Materials and methods section).

Strain	Genotype
W303	*MATα/MATα ade2-1/ade2-1 trp1-1/trp1-, leu2-3, 112/leu2-3, 112 his3-11, 15/his3-11, 15, ura3-1/ura3-1*
W303α	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1*
Δ*inp51*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1, inp51::URA3*
Δ*inp52*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 inp52::HIS3*
Δ*inp53*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 inp53::TRP1*
Δ*inp51*Δ*inp52*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 inp51::URA3, inp52::HIS3*
Δ*inp51*Δ*inp53*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 inp51::URA3, inp53::TRP1*
Δ*inp52*Δ*inp53*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 inp51::HIS3, inp53::TRP1*
Δ*inp51*Δ*inp52*Δ*inp53*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 inp51::URA3, inp52::HIS3, inp53::TRP1*
Δ*inp51*Δ*inp52*Δ*inp53 5-ptase II*	*MATα ade2-1 trp1-1 leu2-3, 112 his3-11,15 ura3-1 inp51::URA3, inp52::HIS3, inp53::TRP1, pRS416:5-phosphatase II*

p415Gal1 plasmid [26]. This plasmid is centromeric and carries the *LEU2* marker gene. This construct was transformed into a diploid triple knockout (Δ*inp51*Δ*inp52*Δ*inp53*) strain, and Leu+, Ura+, His+, Trp+ transformants were selected. Haploid cells were obtained by sporulation and maintained in selective minimal medium for use in the studies described below. The 5-phosphatase II PCR product was sequenced to confirm fidelity of the PCR reaction.

Strains and culture conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. The Δ*inp51*Δ*inp52*Δ*inp53* triple mutant with 5-phosphatase II expression under control of the GAL1 promoter is described as the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II repressed (glucose) or induced (galactose). Cells were cultured in minimal medium lacking the appropriate nutrients to maintain selection conditions for genetic markers. The carbon source utilized was 2% (w/v) glucose, except when induction of the GAL1 promoter was necessary, in which case galactose was used at 4% (w/v).

Northern blot

Total RNA was extracted from galactose-induced or glucose-repressed Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II *S. cerevisiae* at early-exponential phase ($D_{600} \sim 0.6$) by the hot acid/phenol method [27]. A portion of 200 μg of total RNA was denatured and loaded on to a 1% (w/v) agarose gel containing 0.67% formaldehyde, and electrophoresed at 80 V for approx. 3 h. The RNA was transferred on to GeneScreen Plus (NEN) for 18 h and fixed by baking at 80 °C for 2 h. The membrane was prehybridized for 2 h in 0.9 M NaCl, 0.05 M Na_2PO_4, 5 mM EDTA, 5 × Denhardt's solution, 50% formamide and 1% SDS at 42 °C before addition of the denatured cDNA probe. The 5-phosphatase II cDNA was digested with *Apa*L1 and *Dra*III to generate a 797 bp fragment, which was purified (Bresaclean; Bresatec, Adelaide, Australia) and labelled with [α-^{32}P]dCTP by random primers using the High Prime kit (Boehringer, Mannheim, Germany). Hybridization of the probe was performed for 18 h at 42 °C, followed by washing twice (10 min each) with 2 × SSC (0.3 M NaCl and 0.03 M trisodium citrate)/0.1% SDS, and then twice with 1 × SSC/0.1% SDS at the same temperature. After autoradiography for between 4 h (see Figure 1) and 4 days (to ensure no signal in the glucose-repressed lane), the membrane was allowed to decay for 2 months and then reprobed with an end-labelled oligomer yeast actin probe. A 100 ng portion of the yeast actin oligonucleotide probe (ccataccgaccatgataccttggtgtcttg) was end-labelled with [γ-^{32}P]-dATP and T4 polynucleotide kinase, and the labelled probe was added to the membrane, which was processed as before. Washing procedures were repeated and autoradiography was performed.

Analysis of growth characteristics in liquid culture

Starter cultures (10 ml) of yeast strains in minimal medium were grown to early exponential phase ($D_{600} \sim 0.6$) at 28 °C, and then 1 ml was diluted in 100 ml of fresh minimal medium. Cultures were incubated at 28 °C with constant aeration at ~ 200 rev./min. Growth was monitored by measuring D_{600} every 4 h for up to 36 h, and then every 12 h for up to 5 days. Viability was determined by the exclusion of 0.05% New Methylene Blue (Malinckrodt). Aliquots of 1 ml were taken daily for fixation, prior to actin and chitin staining.

Electron microscopy

Mid-exponential-phase cultures of *S. cerevisiae* strains were fixed by addition of phosphate-buffered fixative (final concentrations 1% paraformaldehyde and 1% glutaraldehyde) for 5 min. After centrifugation (1000 ***g***; 10 min), the pellet was resuspended in fresh phosphate-buffered 1% paraformaldehyde/1% glutaraldehyde for a further 30 min. Following a series of rinses in PBS, cells were resuspended in 1% aqueous sodium metaperiodate to assist in permeabilizing the cell wall. A second series of rinses in PBS preceded further fixation in 0.5% aqueous potassium permanganate. Yeast were then washed thoroughly with distilled water, stained *en bloc* with 2% aqueous uranyl acetate, and given an extended wash in distilled water. Cells were dehydrated through a graded series of alcohols and embedded in LR White acrylic resin. Ultra-thin sections were prepared on an Ultracut E Ultramicrotome (Leica), stained with methanolic saturated uranyl acetate and Reynolds lead citrate, and viewed in an Hitachi H600 transmission electron microscope.

Staining of actin and chitin

Yeast cells were fixed by the addition of 0.1 ml of formalin (BDH) solution to 0.9 ml aliquots of growth medium for 30 min, followed by resuspension in formalin/PBS [35 mM K_2PO_4, 35 mM KH_2PO_4, pH 6.5, 5 mM $MgCl_2$ (PBSM)] and 4% formaldehyde for 2 h. The cells were washed once and resuspended in 1 ml of PBS containing 1% Triton X-100. Actin was stained by addition of 0.3 μM phalloidin/TRITC (tetramethylrhodamine

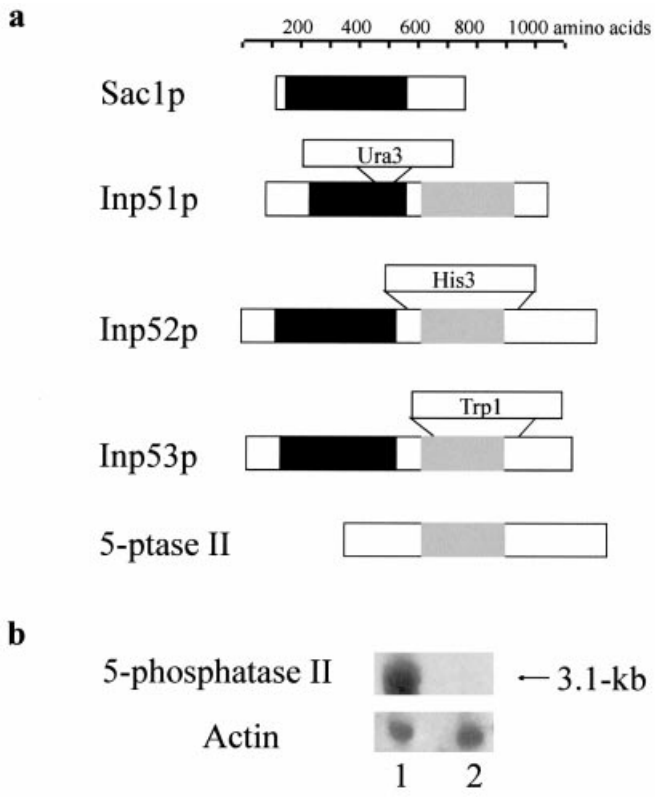

Figure 1 Comparison of the structure of the yeast 5-phosphatases with mammalian 5-phosphatase II

(**a**) Schematic diagram of the disruption of the yeast 5-phosphatases. The three yeast 5-phosphatases are shown with the Sac1-like domain (black) and 5-phosphatase catalytic domain (grey) illustrated, with comparison with the yeast protein Sac1p and the mammalian 5-phosphatase II (5-ptase II). The sites of disruption of each protein are demonstrated by the replacement of the amino acids between the lines by the indicated selective marker. (**b**) 5-Phosphatase II expression is tightly regulated by the GAL1 promoter. Northern blot analysis was performed using 200 μg of total RNA extracted from the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain cultured in the presence of 4% (w/v) galactose (lane 1) or 2% (w/v) glucose (lane 2). Autoradiography for the 5-phosphatase II probe was performed for 4 h, and extended to 4 days to ensure that no signal developed in the glucose-repressed lane. The membrane was allowed to decay and, to confirm similar loading of RNA in each lane, was re-probed with a ^{32}P-labelled actin oligonucleotide and autoradiographed for 3 days.

β-isothiocyanate) (Sigma) for 3 h. Excess stain was removed by washing twice in PBSM. Cells were visualized on poly(L-lysine)-coated slides using confocal microscopy. Chitin staining was performed by addition of 5 μl of calcofluor (1 mg/ml) to 100 μl of fixed yeast cells for 5 min. Excess stain was removed and cells were resuspended as above. The cells were visualized using a Nikkon Optiphot microscope and a UV-2A filter (excitation 330–380 nm; barrier filter 420 nm).

Analysis of cellular Ins(1,4,5)P_3 and PtdIns(4,5)P_2 concentrations

Samples for assay were prepared by trichloroacetic acid extraction of yeast cultures grown to early exponential phase (D_{600} ~ 0.6), as described previously [28,29]. Essentially, 50 ml cultures of yeast were harvested by centrifugation at 1000 ***g*** and washed twice in water, prior to resuspension in 2 ml of PBS. Then 1 ml of culture was lysed with an equal volume of ice-cold 1 M trichloroacetic acid and vortex-mixed with glass beads to aid mechanical disruption. This was followed by incubation on ice for 15 min with intermittent vortex mixing. The solid phase was removed by centrifugation at 4000 ***g*** for 15 min at 4 °C, and used for extraction of PtdIns(4,5)P_2. The supernatant containing the soluble Ins(1,4,5)P_3 was extracted three times with 10 ml of water-saturated diethyl ether, and the pH of the supernatant was adjusted to 7.5 with $NaHCO_3$. The cell pellet was washed with 2 ml of 1 M trichloroacetic acid/1 mM EDTA, followed by distilled water. PtdIns(4,5)P_2 was extracted from this pellet by addition of 0.94 ml of methanol/chloroform/HCl (80:40:1, by vol.) for 15 min at room temperature, with intermittent vortex mixing. The lipid and aqueous phases were resolved by the addition of 0.31 ml of chloroform and 0.56 ml of 0.1 M HCl, followed by centrifugation at 1000 ***g*** for 15 min. Then 400 μl of the lipid phase was evaporated under nitrogen gas, prior to alkaline hydrolysis of the PtdIns(4,5)P_2 to Ins(1,4,5)P_3. A 0.25 ml portion of 1 M KOH was added to the dried lipid and heated at 100 °C for 15 min. Excess alkali was removed by filtration through Dowex 50 (200–400 mesh; H^+ form). The eluate was washed with 2 × 2 ml of butan-1-ol/light petroleum ether (5:1, v/v) before lyophilization. The samples for Ins(1,4,5)P_3 and PtdIns(4,5)P_2 analysis were assayed using the Inositol-1,4,5-Trisphosphate [^{3}H] Radioreceptor Assay Kit (NEN) according to the manufacturer's instructions.

HPLC analysis of *myo*-[^{3}H]inositol-labelled yeast strains

For *myo*-[^{3}H]inositol labelling studies (five population doublings), 6×10^4 cells/ml were inoculated into inositol-free minimal medium (Bio101) containing 10 μCi/ml *myo*-[^{3}H]inositol. Culture conditions were maintained until the cell count reached (1–2) × 10^6 cells/ml (time 8–24 h, depending on strain), which equates to five population doublings. In additional studies, to ensure ^{3}H labelling of all phosphoinositide pools, yeast strains were labelled with *myo*-[^{3}H]inositol for 72 h using the same inoculation conditions.

Lipids were extracted from *myo*-[^{3}H]inositol-labelled yeast strains and deacylated, prior to HPLC analysis by established methods [30]. HPLC analysis was performed using a Zorbax SAX column (4.6 mm × 250 mm; 5 μm) with the following protocol and reagents: buffer A, distilled water; buffer B, 1.25 M $(NH_4)_2HPO_4$, pH 3.8; pump program: 0% B at 0 min; 5 min at 0% B; 65 min at 12% B; 110 min at 80% B.

RESULTS

We investigated the role of PtdIns(4,5)P_2 in mediating the phenotypes observed in double and triple null mutant strains of yeast that lack the Sac1-like-domain-containing 5-phosphatases Inp51p, Inp52p and Inp53p (Figure 1a). We compared the phenotypes of the single, double and triple yeast 5-phosphatase null mutants, in which the 5-phosphatase domain was disrupted, with that of a triple null mutant strain rescued by the mammalian 5-phosphatase II. The latter 5-phosphatase does not express polyphosphoinositide phosphatase activity, and the principal substrate of this mammalian homologue is PtdIns(4,5)P_2. The substrates of 5-phosphatase II include PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. However, yeast do not synthesize PtdIns(3,4,5)P_3, while Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 do not constitute major signalling pathways in yeast, compared with mammalian cells [31,32].

As shown by previous studies, the yeast triple null mutant Δ*inp51*Δ*inp52*Δ*inp53* resulted in a lethal phenotype, as determined by tetrad analysis (results not shown). We transformed this triple null mutant strain with a cDNA encoding a mouse 5-phosphatase II (nucleotides 108–3203) under the control of the GAL1 promoter (Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II). This cDNA

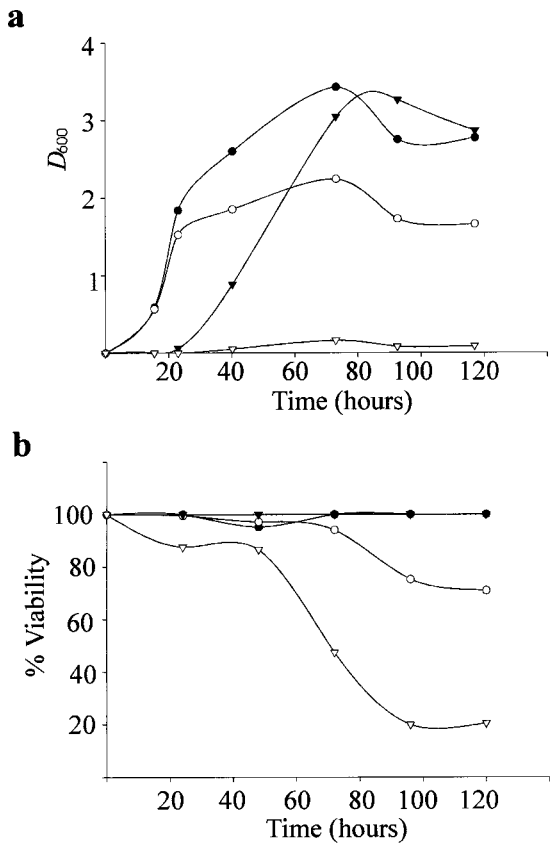

Figure 2 Murine 5-phosphatase II can correct the lethal effects resulting from disruption of all three Sac1-like-domain-containing 5-phosphatases in yeast

Mid-exponential phase starter cultures were diluted 1:100 (v/v) in selective media and incubated at 28 °C. (**a**) D_{600} was monitored to determine the growth of the wild type (●), the Δ*inp51*Δ*inp52* double mutant (○), and the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II triple mutant induced in galactose (▼) or repressed in glucose (▽). (**b**) Viability was determined by exclusion of New Methylene Blue (0.05%) present in the culture for wild type (●), Δ*inp51*Δ*inp52* (○), and Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II repressed (▽) or induced (▼).

encodes a spliced isoform of murine 5-phosphatase II which does not encode a C-terminal CAAX motif, but does encompass the entire 5-phosphatase domain and therefore should localize predominantly to the cytosolic fraction. The expression of the mammalian 5-phosphatase II following induction with galactose was demonstrated by Northern analysis (Figure 1b). In the presence of glucose (2%, w/v), expression was suppressed and no 5-phosphatase II transcript was observed. In contrast, following induction with galactose (4%, w/v), a 3.1 kb transcript was detected in yeast lysates, consistent with the predicted size of the 5-phosphatase II transcript. In addition, immunoblot analysis using mammalian 5-phosphatase II anti-peptide antibodies demonstrated the expression of a 93 kDa polypeptide in galactose-induced yeast cell lysates that was not detected in glucose-treated cells (results not shown). Attempts to determine the intracellular location of murine 5-phosphatase II expressed in yeast using affinity-purified antibodies to this isoform were unsuccessful due to significant non-specific staining.

Growth characteristics of 5-phosphatase null mutant yeast

To determine if 5-phosphatase II was functional in yeast and could rescue the lethal triple null mutant 5-phosphatase phenotype, we analysed the growth characteristics of the wild type, a representative double mutant Δ*inp51*Δ*inp52*, and the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain, both induced (galactose), or repressed (glucose) (Figure 2a). The double Δ*inp51*Δ*inp52* mutant grew at a similar initial rate to wild-type yeast, but did not reach as high an attenuance at saturation. The Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain cultured in the presence of glucose demonstrated no growth. In contrast, when cultured in the presence of galactose, the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain demonstrated a prolonged lag phase of approx. 20 h, before rapid exponential growth was achieved; saturation was reached at an attenuance comparable with that of the wild type. Consistent with this analysis, the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain was inviable when cultured in glucose: within 4 days, 80% of the cells had died (Figure 2b). In contrast, following induction with galactose, the viability of the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain was similar to that of the wild type, and showed enhanced survival compared with Δ*inp51*Δ*inp52*. These results suggest the 5-phosphatase domain from a mammalian enzyme, in the absence of a Sac1-like domain, can function in yeast to rescue the lethal 5-phosphatase triple null mutant phenotype.

Phenotypes of 5-phosphatase null mutant strains

We undertook ultrastructural studies using electron microscopy to compare the phenotypes of 5-phosphatase null mutant strains with those rescued by mammalian 5-phosphatase II (Figures 3a–3f). Wild-type *S. cerevisiae* contain a single nucleus, with usually a single large vacuole or occasionally several smaller vacuoles, a regular plasma membrane and constant cell wall thickness. As reported previously, Δ*inp51*Δ*inp52* double mutants demonstrate grossly abnormal morphology, characterized by a markedly thickened cell wall, fragmentation of vacuoles and an irregular plasma membrane with massive invaginations (Figures 3c and 3d) [17,19,19a,21]. The Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain induced with galactose demonstrated a variable phenotype, with many cells showing a near wild-type morphology (Figure 3f). However, several cells demonstrated structural changes comparable with those in the double null mutant strain, with a grossly thickened cell wall (Figure 3e). The cDNA encoding 5-phosphatase II was cloned into a centromeric plasmid that limits plasmid numbers to a single copy in each cell. Induction of the GAL1 promoter will lead to high levels of 5-phosphatase II expression, but there may be some cellular variation in expression of this enzyme. It was noteworthy that most cells expressing 5-phosphatase II in the triple null mutant strain demonstrated a significantly improved morphology compared with the Δ*inp51*Δ*inp52* double mutant. In general, the cell wall was thinner, vacuoles were larger and less numerous, and the plasma membrane invaginations were less apparent in the majority of cells.

Correction of actin polarization and budding polarity defects by mammalian 5-phosphatase II expression

Defects in actin organization and bud polarity in 5-phosphatase double null mutants have been described in several studies [19,19a,21]. In normal vegetative yeast, actin is localized to patches that are distributed randomly throughout the cortex. The double mutant Δ*inp51*Δ*inp52* showed heterogeneous patch size and large accumulations of actin (Figure 4c). The repressed Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II was grossly abnormal, with very large accumulations of cortical actin (Figure 4g). In contrast, the induced Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II revealed a normal distribution of actin, similar to that in wild-type yeast (Figure 4e). Actin distribution within budding yeast cells depends on the

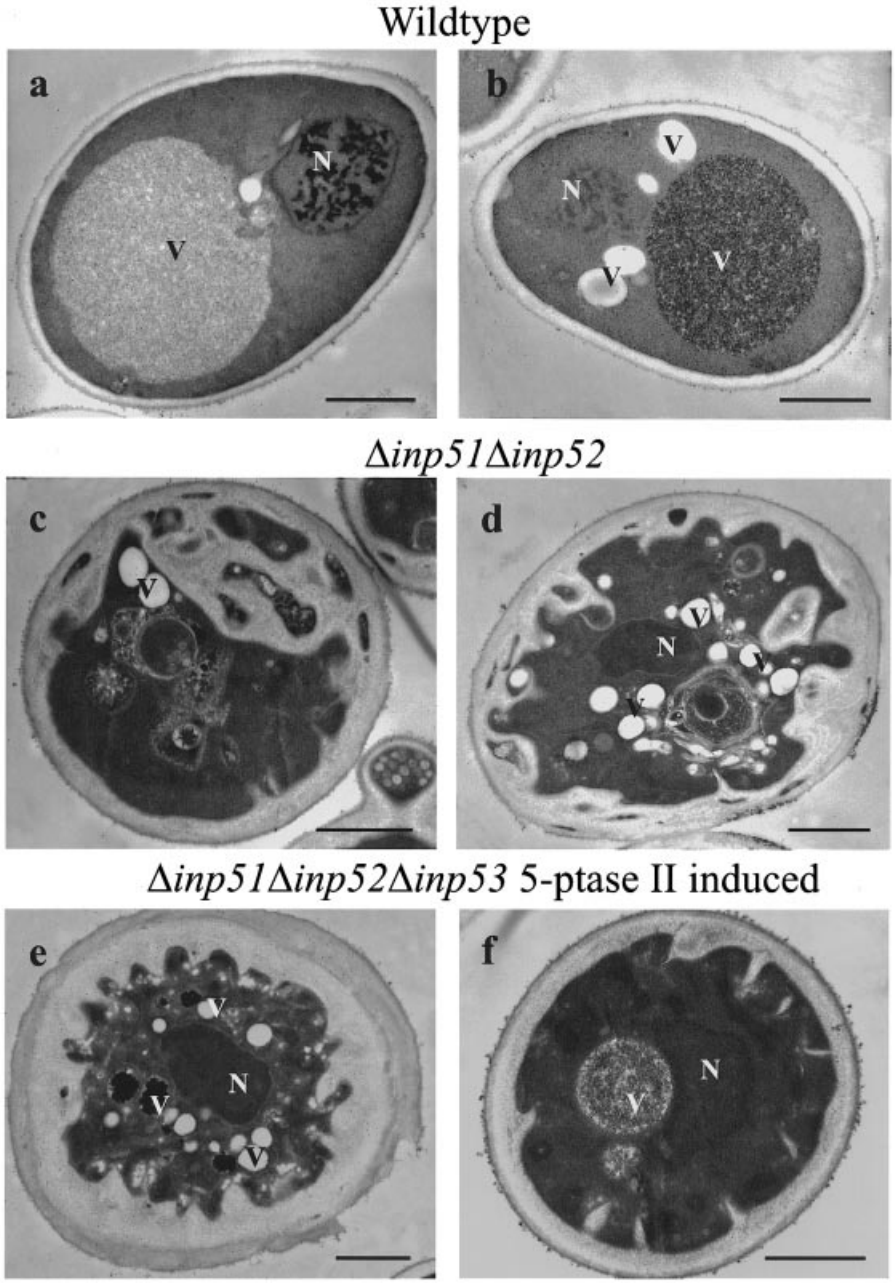

Figure 3 The ultrastructure of triple null mutant yeast is grossly abnormal, and can be rescued by the expression of murine 5-phosphatase II

Electron micrographs of yeast strains were prepared as described in the Materials and methods section. Scale bars represent 1 μm. Panels (**a**) and (**b**) illustrate wild-type yeast, panels (**c**) and (**d**) show representative examples of the grossly abnormal appearance of Δ*inp51*Δ*inp52*, and panels (**e**) and (**f**) demonstrate the heterogeneity of the ultrastructural morphology of the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced strain. The majority of cells appeared as shown in (**f**).

stage of the cell cycle. Early in the budding process, actin patches predominate in the bud, and actin cables within the mother cell are oriented towards the bud. As the bud size increases, actin localizes within both cells, until, at cytokinesis, the actin patches distribute around the bud neck. The Δ*inp51*Δ*inp52* double mutant demonstrated an aberrant distribution of actin, which was present both in the mother cell and in the bud in large patches (Figure 4d). The number of budding cells within the repressed Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II was minimal; however, a grossly aberrant actin distribution was apparent (Figure 4h). In contrast, the induced Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II showed a completely normal actin distribution in most budding cells, with correction of the actin polarity defects. Occasional budding yeast demonstrated mild aberrations in actin morphology, but these were rare.

Calcofluor stains chitin, a cell wall component that is present in 'bud-scars'. In normal haploid cells, new bud-sites are selected at a site adjacent to previous bud-sites, and chitin rings mark the sites where scission occurred. The Δ*inp51*Δ*inp52* mutant showed much brighter staining than the wild type, and the process of

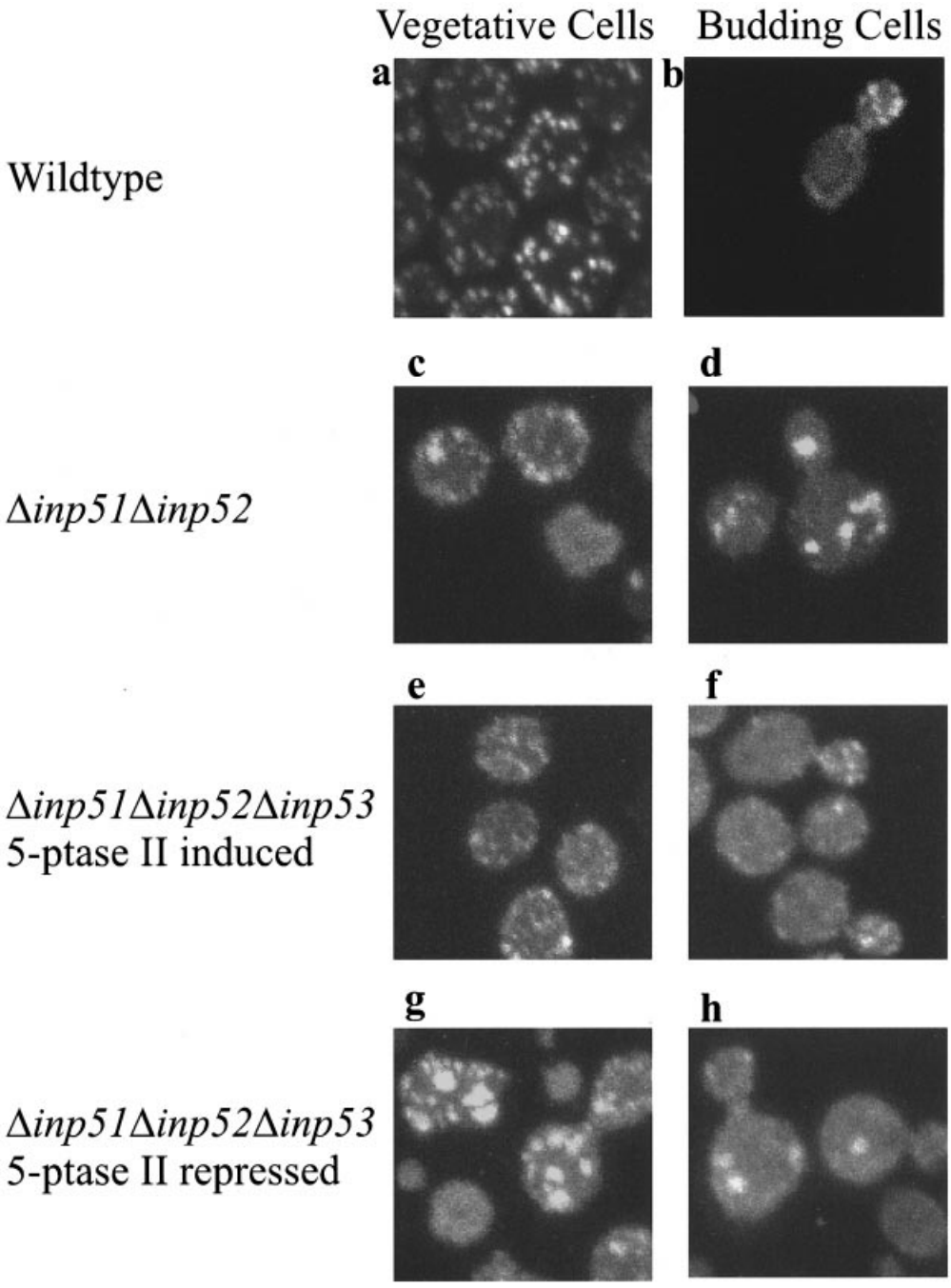

Figure 4 Mammalian 5-phosphatase II can correct the actin localization defect in the Δ*inp51*Δ*inp52* strain

Rhodamine/phalloidin was used to demonstrate the actin distribution within wild-type and 5-phosphatase mutant yeast strains. Representative vegetative and budding cells of the wild type strain (**a**, **b**), Δ*inp51*Δ*inp52* (**c**, **d**) and Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced (**e**, **f**) or repressed (**g**, **h**) are illustrated.

bud-site selection appeared random (Figure 5b). The repressed Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II mutant demonstrated grossly abnormal bud-site selection, with massive overproduction of chitin (Figure 5d). The induced Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain (Figure 5c) showed increased staining, but to a much lesser extent than either the Δ*inp51*Δ*inp52* or repressed Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strains.

Collectively, these studies demonstrate that expression of mammalian 5-phosphatase II, a PtdIns(4,5)P_2 5-phosphatase, can correct the defects in cell growth, actin polymerization, cell wall thickness, vacuole fragmentation and bud-site selection observed in yeast 5-phosphatase null mutant strains.

Phosphoinositide levels in 5-phosphatase null mutant yeast

Several studies have reported the phenotype of the 5-phosphatase null mutants and determined phosphoinositide levels using a variety of techniques. Stolz et al. [18], using TLC, analysed the

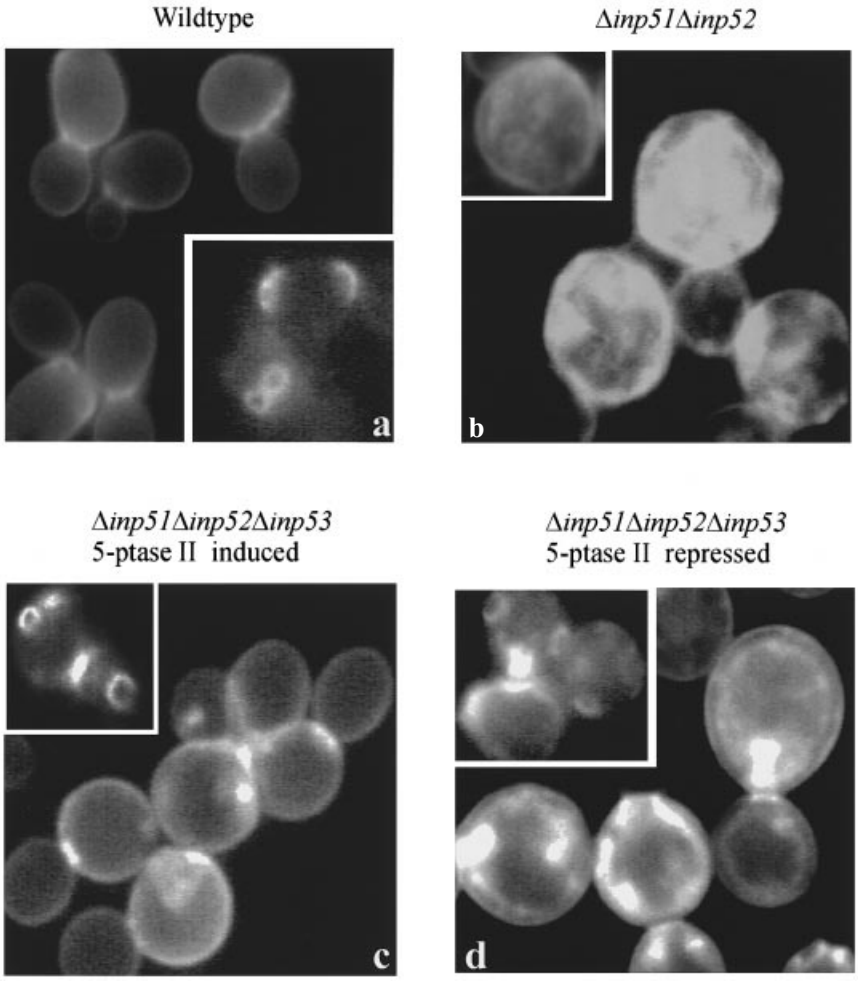

Figure 5 Mammalian 5-phosphatase II corrects the overproduction of chitin and abnormal bud-site selection in 5-phosphatase null mutant yeast

Calcofluor staining of chitin deposition is shown in wild-type (**a**), Δ*inp51*Δ*inp52* (**b**), Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced (**c**) and repressed (**d**) yeast strains. Each panel contains an inset which demonstrates the localization of bud scars.

levels of phosphoinositides in various mutants; however, this analysis does not separate PtdIns(4,5)P_2 from PtdIns(3,5)P_2. In that study [18], PtdInsP_2 levels were elevated in the Δ*inp51*, Δ*inp51*Δ*inp52* and Δ*inp51*Δ*inp53* mutants by only 2-fold; surprisingly, although the Δ*inp52*Δ*inp53* mutant demonstrates a similar phenotype, no abnormalities in phosphoinositide levels were detected. Further analysis of the Δ*inp52*Δ*inp53* strain using [^{3}H]inositol labelling of yeast and analysis of deacylated lipids by HPLC confirmed there was no accumulation of PtdIns(4,5)P_2 [22]. Comparable HPLC analysis of other double mutants has not been reported.

We undertook a detailed analysis of PtdIns(4,5)P_2 levels in 5-phosphatase null mutant yeast and triple 5-phosphatase null mutant yeast rescued by 5-phosphatase II using three distinct strategies. First, we measured PtdIns(4,5)P_2 concentrations using a radioreceptor Ins(1,4,5)P_3 binding assay, as described previously [28,29]. This assay does not rely on equilibrium labelling of yeast. Secondly, we measured phosphoinositides in [^{3}H]-inositol-labelled yeast grown to mid-exponential phase (five doublings). Finally, yeast were labelled for a more extended period of 72 h to stationary phase. Recent studies have shown that the growth status of the cells when they are harvested may modify the responses of phosphoinositides. For example, PtdIns(3,5)P_2 levels are only induced in exponentially growing cells that are subjected to hyperosmotic stress [30]. In addition, several studies have suggested that phosphoinositides may be compartmentalized into several distinct compartments – agonist-sensitive and -insensitive [33]. Heterogeneous pools of PtdIns(4,5)P_2 exist within the plasma membrane of cells. In mammalian cells, PtdIns(4,5)P_2 has been shown to associate with other signalling molecules within membrane microdomains formed by lateral association of sphingolipids and cholesterol [34]. Lipid rafts have also been demonstrated in yeast [35,36]. These microdomains are thought to contain the agonist-sensitive pools of PtdIns(4,5)P_2 that correlate directly with the metabolically labelled pools [33,37]. We reasoned that labelling of exponentially growing yeast may only result in incorporation of [^{3}H]inositol into the rapidly turning over agonist-sensitive phosphoinositide pool, and that more prolonged labelling may be required for incorporation of [^{3}H]inositol into other phosphoinositide compartments.

Total cellular PtdIns(4,5)P_2 and Ins(1,4,5)P_3 concentrations were measured using the Ins(1,4,5)P_3 radioreceptor assay. Endogenous Ins(1,4,5)P_3 and PtdIns(4,5)P_2 were extracted from

Table 2 Total cellular levels of PtdIns(4,5)P_2 and Ins(1,4,5)P_3 in wild-type and 5-phosphatase mutant yeast

Total cellular PtdIns(4,5)P_2 and Ins(1,4,5)P_3 levels were measured using the radioreceptor assay, as described in the Materials and methods section. Values are means ± S.D. for a minimum of three independent assays.

	Concentration (pmol/mg of protein)	
Strain	Ins(1,4,5)P_3	PtdIns(4,5)P_2
Wild type	69 ± 31	147 ± 97
Δ*inp51*Δ*inp52*	53 ± 20	730 ± 226
Δ*inp51*Δ*inp5*Δ*inp53* 5-ptase II induced	83 ± 50	372 ± 296

Table 3 Phosphoinositide levels in wild-type and 5-phosphatase mutant strains following labelling with *myo*-[^{3}H]inositol for five population doublings

Yeast strains were labelled with *myo*-[^{3}H]inositol, as described in the Materials and methods section, for five population doublings (8–24 h). Lipids were extracted and deacylated for analysis by HPLC. GroPtdIns species were identified by comparison with the elution profile of known standards. Mean peak height (c.p.m.) is reported as a percentage of that of GroPtdIns. Values are means ± S.D. for three independent assays. Representative HPLC profiles are illustrated in Figure 6.

	Mean peak height (%)		
Strain	GroPtdIns3*P*	GroPtdIns4*P*	GroPtdIns(4,5)P_2
Wild type	5.34 ± 1.69	2.18 ± 0.17	1.14 ± 0.40
Δ*inp51*Δ*inp52*	5.96 ± 1.18	2.75 ± 1.39	1.44 ± 0.32
Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced	1.57 ± 0.41	1.22 ± 0.34	0.28 ± 0.16

yeast and the PtdIns(4,5)P_2 was converted into Ins(1,4,5)P_3 by alkaline hydrolysis. The concentration of endogenous PtdIns(4,5)P_2 in wild-type yeast was 147 pmol/mg, which was increased to 730 pmol/mg (5-fold increase) in the Δ*inp51*Δ*inp52* mutant (Table 2). In contrast, the galactose-induced Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain demonstrated a significant decrease (2-fold) in PtdIns(4,5)P_2 levels compared with the double null mutant. As the triple knockout is likely to have a greatly elevated endogenous PtdIns(4,5)P_2 level compared with the double mutant, the reduction in PtdIns(4,5)P_2 in the 5-phosphatase rescued triple mutant strain may be even more significant. In contrast, the levels of Ins(1,4,5)P_3 did not appear to differ significantly in the three yeast strains tested (Table 2).

We analysed phosphoinositides in wild-type and 5-phosphatase null mutant yeast by labelling with [^{3}H]inositol for five population doublings, to ensure that > 95% of the total yeast mass was derived from substrates, including radiolabelled inositol, in the growth medium. The lipids were extracted, deacylated to glycerophosphoinositols (GroPtdIns) and analysed by HPLC. The identity of the elution peak was confirmed by comparison with the elution profiles of known standards [GroPtdIns4*P*, GroPtdIns(4,5)P_2 and GroPtdIns(3,5)P_2], and each peak was standardized to the GroPtdIns peak height. PtdIns(4,5)P_2 levels were increased by 1.3-fold in the Δ*inp51*Δ*inp52* mutant, but this was not statistically significant (Table 3; Figure 6). The only previous study analysing PtdIns(4,5)P_2 levels in this strain demonstrated a 2-fold increase in this phosphoinositide; however, TLC analysis rather than HPLC analysis of the deacylated lipid products was used to separate the phosphoinositides, and therefore would not have discriminated between PtdIns(3,5)P_2 and PtdIns(4,5)P_2. In contrast, the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced strain demonstrated a significant decrease in all phosphoinositides compared with the wild type [29% for GroPtdIns3*P*, 56% for GroPtdIns4*P* and 25% for GroPtdIns(4,5)P_2]. Inaccuracies due to varying growth rates were minimized by culturing for a defined number of population doublings. We undertook standardization of peak heights to that of GroPtdIns to minimize variations in labelling or in extraction of phosphoinositides. It is noteworthy that the mean peak of GroPtdIns for the wild type was of a similar order to that obtained for the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced strain (1836438 and

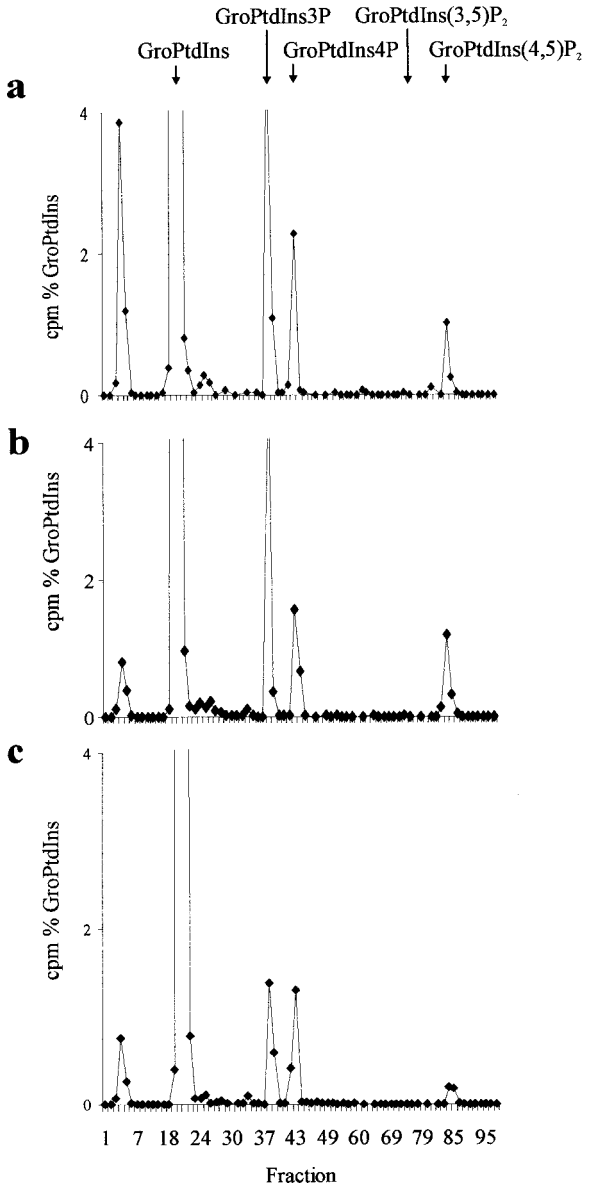

Figure 6 Phosphoinositide levels in wild-type and 5-phosphatase mutant strains following labelling with *myo*-[^{3}H]inositol for five population doublings

Yeast strains were labelled with *myo*-[^{3}H]inositol, as described in the Materials and methods section, for five population doublings (8–24 h). Lipids were extracted and deacylated for analysis by HPLC. GroPtdIns species were identified by comparison with the elution profile of known standards. The mean peak height (c.p.m.) standardized to GroPtdIns is reported in Table 3 as the percentage (mean ± S.D.) for three independent assays. Representative HPLC profiles are illustrated for the wild-type (**a**), Δ*inp51*Δ*inp52* (**b**) and Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced (**c**) strains.

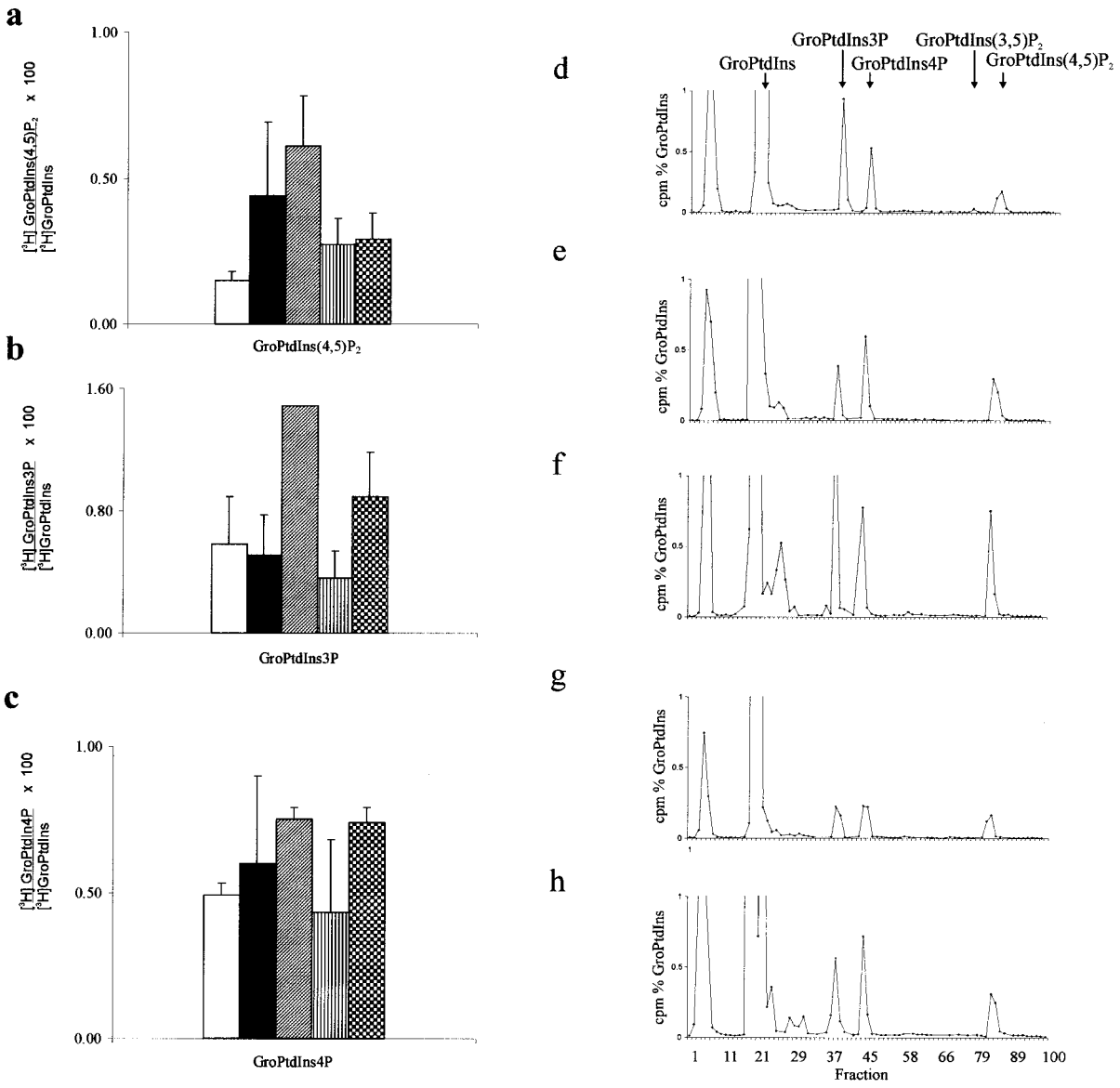

Figure 7 PtdIns(4,5)P_2 levels are elevated in all double mutant 5-phosphatase null strains

Yeast strains were labelled with *myo*-[^{3}H]inositol, as described in the Materials and methods section, for 72 h. Lipids were extracted and deacylated for analysis by HPLC. GroPtdIns species were identified by comparison with the elution profile of known standards. Mean peak height (c.p.m.) standardized to GroPtdIns is reported as the percentage (mean ± S.D. for three independent assays. Endogenous GroPtdIns(4,5)P_2 (**a**), GroPtdIns3*P* (**b**) and GroPtdIns4*P* (**c**) for (from left to right) the wild-type, Δ*inp51*Δ*inp52*, Δ*inp51*Δ*inp53*, Δ*inp52*Δ*inp53* and Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced strains are illustrated. Representative HPLC elution profiles are shown for the wild-type (**d**), Δ*inp51*Δ*inp52* (**e**), Δ*inp51*Δ*inp53* (**f**), Δ*inp52*Δ*inp53* (**g**) and Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced (**h**) strains.

2638589 c.p.m. respectively), suggesting that extraction rates were similar and labelling comparable. In addition, the mass assay used to quantify PtdIns(4,5)P_2 levels in this mutant strain did not demonstrate a decrease in PtdIns(4,5)P_2 levels below those in the wild type (see Table 2). Collectively, these results suggest that the significant decrease in the levels of phosphoinositides, and in particular of PtdIns(4,5)P_2, in the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced strain does not result from reduced levels of endogenous lipids, but is more likely to reflect decreased or delayed synthesis of these phosphoinositides in the agonist-sensitive pool. There were no significant differences noted between the levels of GroPtdIns3*P* or GroPtdIns4*P* in the wild type compared with the Δ*inp51*Δ*inp52* double mutant (Table 3; Figure 6).

To investigate PtdIns(4,5)P_2 levels in 5-phosphatase null mutants further, we increased the time of [^{3}H]inositol labelling in all strains to 72 h (as compared with 8–24 h for five population doublings, dependent on the yeast strain) to ensure saturation labelling (Figure 7). All 5-phosphatase double mutants demonstrated a significant accumulation of GroPtdIns(4,5)P_2, which was most marked (4-fold) in the Δ*inp51*Δ*inp53* double mutant. There was a 3-fold increase in the Δ*inp51*Δ*inp52* strain, but a 2-fold increase was also observed in the Δ*inp52*Δ*inp53* double null mutant, which has not been noted in previous studies [22]. This

analysis suggests that the accumulation of PtdIns(4,5)P_2 may be related to the growth phase of the yeast.

Furthermore, GroPtdIns(4,5)P_2 levels in the galactose-induced Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II strain were 2-fold above those in the wild type, and this showed a significant correction compared with the Δ*inp51*Δ*inp53* double null mutant. It is of interest that, although PtdIns(4,5)P_2 levels were significantly reduced in triple null mutant strains overexpressing 5-phosphatase II to levels observed in the Δ*inp52*Δ*inp53* double null mutant, the phenotype observed in the latter strain (in particular defects in actin polymerization) was more severe (results not shown). These results suggest that it is not the absolute level of PtdIns(4,5)P_2 that is correlated directly with the severity of the phenotype. Localized increases in phosphoinositides in specific membrane compartments may contribute significantly to the observed phenotype.

As the Sac1-like domains of Inp52p and Inp53p hydrolyse PtdIns4*P* and PtdIns3*P*, forming PtdIns, we investigated the levels of PtdIns3*P* and PtdIns4*P* in 5-phosphatase null mutant yeast. Increases of 1.2- and 1.5-fold in GroPtdIns4*P* levels were consistently observed in the Δ*inp51*Δ*inp52* and Δ*inp51*Δ*inp53* null mutant yeast, but no change was noted in the Δ*inp52*Δ*inp53* strain. GroPtdIns3*P* levels were elevated 2.5-fold in Δ*inp51*Δ*inp53* null mutants, but not in any other 5-phosphatase null mutant strains. It was noteworthy that this accumulation of PtdIns3*P* and PtdIns4*P* was not significantly corrected by 5-phosphatase II expression.

DISCUSSION

The study reported here demonstrates that overexpression of a mammalian 5-phosphatase can rescue the lethal phenotype of a triple null mutant yeast 5-phosphatase strain. Overexpression of 5-phosphatase II, a mammalian PtdIns(4,5)P_2 5-phosphatase, corrected defects in actin polymerization and bud-site selection, and partially rescued massive cell wall thickening and fragmented vacuole morphology. We have demonstrated that all yeast 5-phosphatase double null mutant strains are characterized by a significant increase in cellular PtdIns(4,5)P_2 levels. As a result of this analysis, we can assign some of the phenotypic features, in particular defects in actin polymerization, bud-site selection and fragmented vacuole morphology, observed in the 5-phosphatase null mutant strains to increases in PtdIns(4,5)P_2 levels, as this was the only phosphoinositide that demonstrated significant accumulation. However, the absolute cellular level of PtdIns(4,5)P_2 does not correlate directly with specific phenotypes.

In our study, the disruption of *INP52* and *INP53* was designed to occur after the Sac1-like domain (Figure 1a); however, we cannot exclude the possibility that such a truncated protein is unstable, and that the Sac1-like domains of Inp52p and Inp53p are non-functional. It is noteworthy that the phenotype of the 5-phosphatase null mutants that we report is the same as has been described in three previous studies, despite differences in the design of the null mutants. In each study, including ours, the triple null mutant is lethal, while the various 5-phosphatase double null mutants are characterized by a thickened cell wall, vacuole fragmentation, actin polymerization and bud-site selection defects, and abnormalities in endocytosis. In two previously reported studies, the entire open reading frame of each 5-phosphatase was replaced [17,21]. However, in the original study by Srinivasan et al. [19], given the design of the constructs, the possibility cannot be excluded that the Sac1-like domain of Inp51p and Inp52p was functional. The Sac1-like domain of Inp52p and Inp53p hydrolyses PtdIns3*P*, PtdIns4*P* and PtdIns(3,5)P_2; however, no definitive HPLC analysis of the endogenous levels of these phosphoinositides in either the single or double 5-phosphatase null mutants has been reported, except for the Δ*inp52*Δ*inp53* strain [22]. In this latter report, no differences in the levels of PtdIns3*P* or PtdIns4*P* were detected after 10 population doublings, although at early exponential phase the levels of PtdIns(3,5)P_2 were increased 5–7-fold after stress. We also demonstrated no changes in the levels of PtdIns3*P* and PtdIns4*P* in the Δ*inp52*Δ*inp53* null mutant strain; under our prolonged labelling conditions and in the absence of osmotic shock, we would not anticipate detecting changes in PtdIns(3,5)P_2. However, we noted, following prolonged labelling of yeast for 72 h, a significant increase in the levels of PtdIns3*P*, and to a lesser extent PtdIns4*P*, in the Δ*inp51*Δ*inp52* null mutant strain, suggesting that the Sac1-like domain of Inp52p is non-functional and that accumulation of these phosphoinositides may be detected given prolonged labelling conditions. Consistent with this contention, induction of 5-phosphatase II, which does not contain a Sac1-like domain, did not correct the level of these phosphoinositides, but reduced the accumulation of PtdIns(4,5)P_2 and corrected the abnormal phenotype.

Decreased labelling of phosphoinositides in 5-phosphatase null mutant strains

We have demonstrated that the levels of PtdIns(4,5)P_2 detected in 5-phosphatase null mutant strains are variable and dependent on the time period of [^{3}H]inositol labelling. We have shown, using more prolonged labelling, significant increases in the levels of PtdIns(4,5)P_2 of 4.5-, 3- and 2-fold in the Δ*inp51*Δ*inp53*, Δ*inp51*Δ*inp52* and Δ*inp52*Δ*inp53* double null mutants respectively. Previous studies have suggested that either Inp53p [19,19a] or Inp51p [17] is the most active PtdIns(4,5)P_2 5-phosphatase in yeast. In our study, it was noteworthy that the highest levels of endogenous PtdIns(4,5)P_2 were detected in strains in which *INP51* was disrupted, suggesting that this enzyme is the most active of the yeast 5-phosphatases in regulating PtdIns(4,5)P_2 levels, consistent with the report by Stolz et al. [18]. Although all yeast 5-phosphatases have been shown to hydrolyse PtdIns(4,5)P_2, the relative affinity of each isoform for this phosphoinositide has yet to be determined. Also, PtdIns(4,5)P_2 has a proposed signalling function in many membrane compartments, including the inner wall of the plasma membrane, Golgi membranes and the endoplasmic reticulum. The localized regulation of this phosphoinositide in these sites by specific 5-phosphatases is therefore of central importance.

The HPLC analysis of endogenous phospholipids was initially performed on yeast strains in exponential growth labelled for five population doublings. No accumulation of PtdIns(4,5)P_2 was detected in the Δ*inp51*Δ*inp52* strain or the Δ*inp51*Δ*inp52inp53* 5-ptase II induced triple null mutant strain. This was not due to inadequate labelling, as cell counts were used to standardize this parameter, nor was it due to inadequate extraction procedures, as the peak height of [^{3}H]GroPtdIns was of the same order as in the wild type. In contrast, the Δ*inp51*Δ*inp52* strain labelled for 72 h demonstrated a 4-fold accumulation of PtdIns(4,5)P_2 and a 5-fold increase in PtdIns(4,5)P_2 using specific mass assays. The analysis of PtdIns(4,5)P_2 levels after labelling for five population doublings was performed at a very early exponential phase of growth. In contrast, the radioreceptor PtdIns(4,5)P_2 mass assays were performed at an early to mid exponential phase of growth, and the 72 h labelling of [^{3}H]inositols in yeast was performed when the cell culture had achieved saturation. The relative comparison between yeast 5-phosphatase mutant strains demonstrates that there is no significant accumulation of PtdIns(4,5)P_2 in the early exponential phase of growth (after five population

doublings), but that accumulation of PtdIns(4,5)P_2 becomes apparent at mid-exponential phase [radioreceptor PtdIns(4,5)P_2 mass assay] and persists until early saturation phase (72 h [^{3}H]inositol labelling). These results suggest that the action of 5-phosphatases in controlling levels of PtdIns(4,5)P_2 is growth-phase-dependent.

5-Phosphatase regulation of PtdIns(4,5)P_2 levels in the cell

The importance of 5-phosphatase isoforms in the regulation of PtdIns(4,5)P_2 levels in mammalian cells has been demonstrated in several studies. It is noteworthy that cell lines deficient in OCRL (the enzyme deficient in oculocerebrorenal syndrome of Lowe) show a 2-fold accumulation of PtdIns(4,5)P_2, as detected by labelling with [^{3}H]inositol for 3 days in culture, until the cells reach confluence [38]. In addition, synaptojanin-1 knockout mice demonstrate a 2-fold accumulation of PtdIns(4,5)P_2, but, despite the loss of the Sac1-like domain, show no accumulation of PtdIns3*P*, PtdIns4*P* or PtdIns(3,5)P_2 [16]. Maintenance of PtdIns(4,5)P_2 levels has been demonstrated to be critical during the yeast cell cycle. Electroporation of a monoclonal antibody directed against PtdIns(4,5)P_2 into yeast resulted in inhibition of cell growth [39]. The effects of PtdIns(4,5)P_2 on the actin cytoskeleton of eukaryotic cells have been shown to be many and varied, but, in general, lead to strengthening of the actin network via regulation of various actin-binding proteins, such as profilin, gelsolin and CapZ [40,41]. The Δ*inp51*Δ*inp52* double mutant demonstrated an abnormal actin distribution both in vegetative cells, in which the actin patches were larger than in the wild type, and also in budding cells, which showed delocalization of actin patches to the mother cell. The aberrant actin distribution was grossly exaggerated in the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II suppressed strain, but this defect was completely corrected by 5-phosphatase II expression, correlating with a decrease in PtdIns(4,5)P_2 levels. The results of this analysis suggest either that the Sac1-like domain plays no role in mediating the phenotype, or that loss of function of the Sac1-like domain may be compensated by significant increases in PtdIns(4,5)P_2 5-phosphatase activity.

It is somewhat surprising that the Δ*inp52*Δ*inp53* null mutant never demonstrated greater than 2-fold accumulation of cellular PtdIns(4,5)P_2, whereas the phenotype of this mutant reveals the most profound defects in actin polymerization. In addition, although the Δ*inp51*Δ*inp53* mutant showed the greatest accumulation of PtdIns(4,5)P_2 (4-fold), the phenotype of this double mutant is less profound than for the other double null mutants; in particular, actin cytoskeletal abnormalities are not detected, the plasma membrane is only slightly distorted and the vacuolar defects are less apparent [31]. Therefore the cellular accumulation of PtdIns(4,5)P_2 does not correlate directly with the severity of the phenotype, or with specific phenotypes, although overexpression of a mammalian PtdIns(4,5)P_2 5-phosphatase does correct the phenotype. This apparent discrepancy may relate to the specific intracellular location of each 5-phosphatase isoform in yeast. Loss of the localized hydrolysis of PtdIns(4,5)P_2 in distinct subcellular compartments may contribute to the observed phenotype. Alternatively, the 5-phosphatases, via their respective proline-rich domains, form complexes with specific localized signalling networks, as has been shown for mammalian homologue synaptojanin [42–44]. As we have expressed mammalian 5-phosphatase II at high levels, and we predict that this would occur predominantly in the cytosol, the regulation of PtdIns(4,5)P_2 may be corrected in many cellular compartments.

The vacuole in yeast is a dynamic organelle that changes its physical characteristics in response to environmental and nutrient stresses [45], and also during cell division [46]. Changes in vacuolar morphology occur by progressive cycles of fragmentation and fusion of vacuoles, which has been classified into four stages: priming, tethering, docking and fusion [47]. The abnormal vacuole morphology illustrated here for the Δ*inp51*Δ*inp52* double mutant demonstrates multiple small vacuoles, suggesting that fusion is inhibited in this strain. Synthesis of PtdIns4*P* and PtdIns(4,5)P_2, but not PtdIns3*P* or PtdIns(3,5)P_2, has been demonstrated to be crucial in this process [48]. It is noteworthy that the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced mutant strain demonstrated a marked improvement in vacuolar morphology compared with the double null mutant strains. We propose that the improvements in actin organization, chitin deposition and vacuolar morphology in the Δ*inp51*Δ*inp52*Δ*inp53* 5-ptase II induced mutant strain are correlated with the improvement in the cellular levels of PtdIns(4,5)P_2, rather than of other phosphoinositides such as PtdIns3*P*, PtdIns4*P* and PtdIns(3,5)P_2 regulated by the Sac1-like domain.

Finally, recent studies in our laboratory and others have shown that the catalytic mechanism of action of 5-phosphatases is highly conserved between yeast and mammalian isoforms, and resembles that of the apurinic/apyrimidine endonucleases [49,50]. Collectively, the studies reported here indicate evolutionary conservation of function between mammalian and yeast 5-phosphatases, and indicate the cellular consequences of PtdIns(4,5)P_2 accumulation.

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