Pleiotropic effects of the *opi1* regulatory mutation of yeast: its effects on growth and on phospholipid and inositol metabolism

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Key factors which impact on the biosynthesis and subsequent fate of the phospholipid precursor inositol were studied as a function of growth phase in the yeast *Saccharomyces cerevisiae*. Both wild-type and strains disrupted for the *OPI1* gene, the principal negative regulator of the phospholipid biosynthetic genes, were examined. Overexpression of the *INO1* gene and overproduction of both inositol and the major inositol-containing phospholipid, phosphatidylinositol, varied as a function of growth phase. In *opi1* cells, *INO1* expression was constitutive at a high level throughout growth, although the level of transcript was reduced at stationary phase when the cells were grown in defined medium. In the wild-type strain, *INO1* expression was limited to a peak in the exponential phase of growth in cells grown in the absence of inositol. Interestingly, the pattern of *OPI1* expression in the wild-type strain resembled that of its putative target, *INO1*. Intracellular inositol contents of the *opi1* strain were higher than those of the wild-type strain, with peak levels occurring in the stationary phase. Membrane phosphatidylinositol content paralleled intracellular inositol content, with *opi1* strains having a higher phosphatidylinositol content in stationary phase. The proportion of the predominant phospholipid, phosphatidylcholine, exhibited a profile that was the inverse of the phosphatidylinositol content: phosphatidylcholine content was lowest in *opi1* cells in stationary phase. The *opi1* mutation was also found to have effects beyond phospholipid biosynthesis. *opi1* cells were smaller, and *opi1* cultures achieved a cell density twice as high as comparable wild-type cultures. *opi1* cells were also more salt tolerant than wild-type cells: they were partly resistant to shrinking, more rapidly resumed growth, and attained a higher culture density after upshift to medium supplemented with 8% NaCl.

Keywords: *Saccharomyces cerevisiae*, inositol, *OPI1*, *INO1*, growth

INTRODUCTION

Membrane biogenesis is a dynamic process which must be closely coordinated with cell growth and division. This coordination implies that the biosynthesis of membrane components, such as phospholipids and their precursors, must also be regulated during growth. In the budding yeast *Saccharomyces cerevisiae*, inositol 1-phosphate is formed from glucose 6-phosphate in a reaction catalysed by inositol-1-phosphate synthase, the

product of the *INO1* gene. Inositol 1-phosphate is subsequently dephosphorylated to form free inositol. In a reaction catalysed by the membrane-associated enzyme phosphatidylinositol (PI) synthase, inositol is combined with CDP-diacylglycerol to yield PI. In yeast, the phosphorylated versions of PI (PIP and PIP₂), as well as the inositol-containing sphingolipids, are all derived from PI (Angus & Lester, 1972). In higher eukaryotes, turnover of PI, PIP and PIP₂ has been shown to generate the phosphorylated inositides involved in signal transduction (Berridge, 1987). In yeast, these signal transduction pathways are not as well characterized as in mammals (Paltauf *et al.*, 1992).

An important point of control for inositol biosynthesis

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

is the expression of *INO1*, the structural gene encoding inositol-1-phosphate synthase, which catalyses the rate-limiting step of inositol synthesis from glucose 6-phosphate. In yeast, at least three regulatory factors are involved in this control (Ambroziak & Henry, 1994; Nikoloff & Henry, 1994; White *et al.*, 1991). Two positive regulators are encoded by the *INO2* and *INO4* genes (Donahue & Henry, 1981a, b; Loewy & Henry, 1984), whereas the *OPI1* gene encodes a negative regulatory factor (Greenberg *et al.*, 1982a). Cells carrying the *opi1* mutation overexpress *INO1* and synthesize excess inositol which is excreted from the cell via an unknown mechanism and accumulates in the growth medium. This gives rise to the overproduction of inositol (*Opi*⁻) phenotype, which can be detected in a plate assay (Greenberg *et al.*, 1982b).

In wild-type strains, *INO1* is regulated by the *INO2*, *INO4* and *OPI1* gene products in response to the availability of free inositol and a second phospholipid precursor, choline (Hirsch & Henry, 1986). Hence, when exponentially growing wild-type cells are cultured in the absence of inositol and choline, the *INO1* gene is maximally expressed. If these precursors are added to the growth medium, *INO1* expression is repressed in the exponential phase. In *opi1* cells, the *INO1* transcript fails to be repressed in exponential phase in response to inositol and choline (Hirsch & Henry, 1986). In wild-type cells, the level of *INO1* mRNA is also affected by the levels of CDP-diacylglycerol synthase activity (Shen & Dowhan, 1997). Similarly, the *INO1* gene is repressed upon entry into stationary phase even when inositol and choline are absent from the growth medium (Lamping *et al.*, 1995). It is not clear whether stationary-phase regulation of *INO1* occurs by a mechanism separate from or related to the mechanism that controls its repression in response to inositol and choline. In this study, we analysed *INO1* gene expression in *opi1* and wild-type cells and found that stationary-phase repression of *INO1*, like the response to soluble precursors, depends upon the *OPI1* gene product. Furthermore, we found that the *opi1* mutation also affects growth and cell morphology, suggesting that the *OPI1* gene product plays a role in control of cell growth that is more global than previously supposed.

METHODS

Strains, growth conditions, transformations and recombinant DNA techniques. *Escherichia coli* DH5 α was grown at 37 °C on LB medium and transformed as previously described (Hanahan, 1983). Selection of transformants or propagation of plasmids was achieved using LB medium containing ampicillin at 50 μ g ml⁻¹.

The genotypes of the strains of *S. cerevisiae* used in this study are detailed in Table 1. Precultures were grown by inoculating one-third of a loopful of yeast into 15 ml YEPD (1% yeast extract, 2% peptone, 2% glucose) and grown at 30 °C to an optical density of approximately 1000 Klett units (640 nm filter; approx. $2-4 \times 10^8$ cells ml⁻¹). Experimental cultures were inoculated from YEPD precultures to an optical density of 20 Klett units (approx. $4-8 \times 10^6$ cells ml⁻¹). The inositol-free (I⁻) synthetic complete medium used in all experiments

has been reported elsewhere (Culbertson & Henry, 1975). Cultures were grown in 125 ml nephelometer side-arm flasks to allow direct measurement of cell growth using a Klett-Summerson colorimeter. Cell numbers were determined from microscopic counts of diluted samples using a haemocytometer.

Yeast transformations were carried out by the method of Ito *et al.* (1983) and transformants were selected on synthetic complete medium lacking the appropriate amino acid. Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989).

Plasmid and strain construction. Plasmid pVJ103 was prepared by ligating a 3932 bp *SspI*-*NsiI* fragment containing an *INO1-lacZ* reporter fusion from pJH334 (Hirsch, 1987; Lopes & Henry, 1991) into the 3976 bp *AatII*-*SphI* fragment from plasmid pBJ6896 (Jones & Prakash, 1990). The congenic strains JAGWT (wild-type) and JAG1 (*opi1* Δ) containing the *his3*, *leu2*, *trp1* and *ura3* auxotrophic markers were produced by crossing OP- Δ 2 with W303-1B (Table 1).

The prototrophic strains WT-*lacZ* (wild-type) and OP-*lacZ* (*opi1* Δ) were constructed from strain DC5 and its *opi1* derivative, OP- Δ 2, respectively (White *et al.*, 1991). To first restore leucine prototrophy, DC5 was transformed with an *SspI* fragment from YE351 (Hill *et al.*, 1986) containing 1440 bp of the *LEU2* gene. Subsequently, both this derivative of DC5 and OP- Δ 2 were transformed with pVJ103 digested to completion with *XhoI*. Successful transformants were identified by the simultaneous restoration of histidine prototrophy and the presence of the *INO1-lacZ* fusion as demonstrated by Southern blot analysis (Southern, 1975), probing with labelled pVJ103 linearized with *XhoI*. The *INO1-lacZ* fusion was utilized in initial experiments to provide a means for the ready estimation of *INO1* expression.

Osmotic shock experiments. The wild-type (WT-*lacZ*) and *opi1* (OP-*lacZ*) strains were grown in I⁻ medium until the cultures had reached the late exponential/early stationary phase of growth (approximately 24 h). Samples of these cultures were then diluted 25-fold into fresh I⁻ medium or I⁻ medium supplemented with NaCl to 8% (w/v). Optical density (640 nm) of the cultures was monitored at various time intervals over approximately 70 h after the shift. A stage micrometer was used to determine the longest dimension of 35 randomly selected mother cells or unbudded single cells at each sample point.

Extraction of intracellular fraction. Cell extracts for the determination of intracellular inositol content were prepared from culture samples of wild-type and *opi1* cells containing the equivalent of 2000 Klett units ml⁻¹ of cells (approx. $4-8 \times 10^8$ cells). Samples were chilled on ice, and the cells were harvested by centrifugation at 4 °C, washed with cold 0.1 M KCl solution and resuspended in 2 ml distilled water. Four cycles of freezing (-80 °C) and boiling for 15 min were used to permeabilize the cells. Following this treatment, cellular debris was removed from samples by centrifugation at 4000 g for 5 min. Supernatants were then assayed in triplicate according to the bioassay outlined below.

Bioassay determination of inositol concentration. A suspension of the inositol-requiring AID tester strain (see Table 1 for genotype) was prepared by growing it in YEPD medium supplemented with adenine to 50 mg l⁻¹ and inositol to 12 mg l⁻¹. Cells were harvested by centrifugation and washed four times with equal volumes of sterile distilled water. Washed cells were resuspended in sterile distilled water to an optical density of approximately 500 Klett units (approx. $1-2 \times 10^8$ cells ml⁻¹) and stored at 4 °C.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
DC5	MATa <i>leu2-3,-112 his3-11,15</i>	J. Broach
W303-1A	MATa <i>ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura2-1</i>	R. Rothstein
W303-1B	MATa <i>ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura2-1</i>	R. Rothstein
OP-Δ2	MATa <i>opi1-Δ::LEU2 leu2-3,-112 HIS3</i> [pVJ103, <i>INO1'</i> lacZ] <i>his3-11,15</i>	White <i>et al.</i> (1991)
WT-lacZ	MATa <i>HIS3</i> [pVJ103, <i>INO1'</i> lacZ] <i>his3-11,15</i>	This study
OP-lacZ	MATa <i>opi1-Δ::LEU2 leu2-3,-112 HIS3</i> [pVJ103, <i>INO1'</i> lacZ] <i>his3-11,15</i>	This study
JAGWT	MATa <i>his3 leu2 trp1 ura2</i>	This study
JAG1	MATa <i>his3 leu2 trp1 ura3 opi1-Δ::LEU2</i>	This study
AID	MATa/MATa <i>ade1/ade1 ino1/ino1</i>	Greenberg <i>et al.</i> (1982b)

Samples of cell extracts of wild-type and *opi1* strains (400 µl) were prepared as described in the preceding section and added to 15 ml tubes containing 5 ml I⁻ medium and autoclaved. Once cooled, all tubes were inoculated with 100 µl of the tester strain suspension and incubated in a tube roller at 30 °C for 36 h. Standards containing known amounts of inositol in the range 0–22 µg were inoculated and incubated in the same fashion, and cell density was quantified at the end of the incubation, as optical density (Klett units). The inositol content of the experimental samples was calculated by comparing the growth supported by the sample to the growth supported by the corresponding quantity of inositol on the standard curve.

RNA analysis. RNA probes for Northern blot hybridization were synthesized using an SP6/T7 Transcription Kit (Boehringer Mannheim) according to the manufacturer's instructions. An *INO1* riboprobe was prepared from a T7-driven transcription of plasmid pJH310 (Hudak *et al.*, 1993) linearized with *Hind*III. A riboprobe specific for the *TCM1* gene was prepared from an SP6-driven transcription of pAB309Δ linearized with *Eco*RI. The *OPI1* probe was prepared from plasmid pJAG15 (Graves, 1996), which consists of a 1.3 kb *Spe*I–*Nsi*I fragment of *OPI1* (White *et al.*, 1991) carried in pGEM9Zf(–) (Promega). The *OPI1* riboprobe was prepared using SP6-driven transcription, linearized with *Eco*RI. Radiolabelled riboprobe was purified from the reaction mix chromatographically (Stratagene). RNA was isolated from yeast using glass-bead disruption and hot phenol extraction (Elion & Warner, 1984). Total RNA concentrations were estimated spectrophotometrically. Northern hybridization was performed as described by Hirsch & Henry (1986). Relative loading of total RNA was determined by photoimaging and quantification of the intensities of the two rRNA bands visualized in each sample following methylene blue staining (Melton *et al.*, 1984). An Ambis 4000 phosphor-imager and associated software were used to optically image agarose gels or blots. rRNA bands were visualized either by UV illumination of ethidium-bromide-stained gels before transfer to nitrocellulose or by methylene blue staining of nitrocellulose-bound RNA (Sambrook *et al.*, 1989). Autoradiographs were developed and quantified using the phosphorimager.

Phospholipid composition. Cultures were grown in I⁻ medium containing 1.85 × 10⁵ Bq of [³²P]orthophosphate ml⁻¹. Pre-cultures were grown in YEPD containing the same amount of label as the main culture to ensure uniform labelling of early

time-point samples. Lipids were extracted as described by Atkinson *et al.* (1980) from culture samples containing the equivalent of 400 Klett units ml⁻¹ (approx. 0.5–1 × 10⁸ cells). Lipids were resolved by two-dimensional paper chromatography on silica-impregnated paper using the method of Steiner & Lester (1972). Labelled spots corresponding to specific lipids were imaged and quantified using a phosphor-imager and quantification software (Ambis). The relative abundance of individual phospholipid species was expressed as a proportion of the total radioactivity in each lipid sample.

RESULTS

Effects of the *opi1* mutation on growth and cell morphology

Isogenic pairs of strains, with and without the *opi1* mutation, were constructed as described in Methods. The growth characteristics of these strains were compared by monitoring the optical density, total cell number and viability of cultures grown in inositol-free (I⁻) medium. When measured by optical density, the growth kinetics of *opi1* strains were not markedly different from the wild-type strain (Fig. 1). The *opi1*

Fig. 1. Growth of wild-type (WT-lacZ; solid symbols) and *opi1* (OP-lacZ; open symbols) in I⁻ medium. Optical density of the culture (●, ○) and total cell numbers (■, □) were determined as described in Methods. Values are the means of two determinations performed in duplicate.

Table 2. Culture biomass attained by wild-type and *opi1* mutant strains at two points during growth

Cells were grown in I⁻ medium and samples were taken when the cultures attained an optical density of 100 Klett units at 9.66 h (exponential phase) and 325 Klett units at approximately 24 h (stationary phase). See Fig. 1 for growth curves.

Growth phase	Wild-type (WT- <i>lacZ</i>)		<i>opi1</i> Δ (OP- <i>lacZ</i>)	
	Dry weight (g l ⁻¹)	Cell mass (pg per cell)*	Dry weight (g l ⁻¹)	Cell mass (pg per cell)*
Exponential	1.44 ± 0.11	48.5	1.48 ± 0.06	32.8
Stationary	3.14 ± 0.07	43.2	2.71 ± 0.12	20.3

* Calculated from the reported dry weights and the cell counts shown in Fig. 1.

Fig. 2. Cell morphology of exponential cultures (18 h after inoculation into I⁻ medium) of (a) wild-type (WT-*lacZ*) and (b) *opi1* (OP-*lacZ*) strains. Bars, 10 µm.

mutation did, however, affect cell number. The *opi1* cultures exhibited approximately twice the cell number observed in wild-type cultures of comparable optical density (Fig. 1). This nearly twofold increase in cell number did not produce a similar increase in biomass formation at either of the two time points examined. Rather, there was a decrease in the mean cell weight in the *opi1* mutant as compared to the wild-type (Table 2). Microscopic examination confirmed that at any specific

Fig. 3. Proportion of budding cells (solid line) and viable cells (broken line) of the total cell population for wild-type (WT-*lacZ*; ●) and *opi1* (OP-*lacZ*; ○) strains. Cultures were grown in I⁻ medium as for Fig. 1. Error bars (SD) are depicted in only one direction. Values are the means of two determinations performed in duplicate.

optical density, cells in *opi1* cultures were more numerous, but that they were also smaller (Fig. 2). Culture viability and the proportion of cells undergoing budding appeared unaffected by mutation of the *OPI1* gene (Fig. 3).

A key response of cells to osmotic shock is a rapid and transient decrease in cell size/volume (Albertyn *et al.*, 1994; Reed *et al.*, 1987; Rose, 1975). The *opi1* strain was evaluated to determine whether its smaller cell size conferred a greater resistance to osmotic shock than is observed for the wild-type strain. A dramatic inhibition of growth was observed in both strains in 8% NaCl medium as compared with the unsupplemented I⁻ medium (Fig. 4a). However, the growth inhibition was less severe in the *opi1* strain, which displayed a shorter lag in growth under these conditions and achieved a higher growth rate and a greater final culture density than the wild-type strain (Fig. 4a). When shifted to fresh medium with no NaCl, cells of both strains showed a rapid increase in size, probably due to initiation of

Fig. 4. Effect of osmotic shock on growth (a) and mean cell diameter (b) of wild-type (WT-*lacZ*; ●) and *opi1* (OP-*lacZ*; ○) strains. Cultures were grown as described in Methods in I⁻ medium and transferred into fresh I⁻ medium with (solid curves) or without (broken curves) supplementation with NaCl to 8% (w/v). Values are the means of two determinations.

growth (Fig. 4b). Original cell sizes were restored for both strains after 3–4 h. However, when the cells were shifted to medium containing NaCl, their sizes were reduced in both strains during the period immediately following the transfer (Fig. 4b). The diameter of wild-type cells was reduced by approximately 15% in less than 30 min following shift to medium containing NaCl, whereas a reduction in cell diameter of the order of 6% was seen in the *opi1* mutant, but only after approximately 2 h. In both strains, a return to pre-shift cell diameter had occurred by 3 h.

Growth-phase-dependent expression of *INO1*

A key target of the *OPI1* gene product (Opi1p) is the *INO1* gene (Bachhawat *et al.*, 1995; White *et al.*, 1991). The expression of *INO1* at various growth phases was investigated in both wild-type and *opi1* strains grown in I⁺ (inositol-containing) and I⁻ media. Cultures were sampled at intervals, total RNA was extracted, and the relative abundance of the *INO1* transcript was determined as described in Methods. In the wild-type strain, *INO1* expression varied in response to both precursor availability and growth phase (Fig. 5). For cultures grown in the absence of inositol, *INO1* transcript in the wild-type strain was most abundant during the period corresponding to exponential growth and undetectable either side of this interval. The *INO1* transcript level was low at all growth stages in cells grown in the presence of inositol. In the *opi1* strain grown under the same conditions, *INO1* transcript was expressed at high levels at all growth stages. Whereas

growth in medium containing inositol repressed expression of *INO1* in the wild-type strain, inositol had no effect on the *opi1* strain, which retained high levels of *INO1* expression throughout all growth phases, regardless of the presence of inositol.

In the wild-type strain, *INO1* expression increased from trace levels at the time of inoculation of the culture (as described in Methods, the inoculum was derived from an early stationary-phase culture growing in YEPD medium) to peak expression during the exponential phase of growth in I⁻ medium (Fig. 6). Expression then again declined to trace levels by stationary phase. In contrast, at the time of inoculation, the *opi1* culture displayed a high level of *INO1* transcript (Fig. 6). During active growth in I⁻ medium, the level of *INO1* transcript in the *opi1* strain gradually declined to a level similar to the maximum derepressed level seen for the wild-type strain during exponential phase (Fig. 6). As the cells entered stationary phase, *INO1* transcript abundance in the *opi1* strain declined, but to a level only slightly less than the maximum derepressed level seen in the wild-type cells. In *opi1* cells, the *INO1* transcript was never fully repressed.

Regulation of the *OPI1* gene

Northern analysis of the *OPI1* gene revealed a single transcript of approximately 1.6 kb (data not shown). To assess the manner in which the *OPI1* gene was regulated, RNA samples were harvested from cells grown to various growth phases in both I⁺ and I⁻ media. These RNA samples were used for slot-blot analysis (Fig. 7). An increase in *OPI1* transcript was observed when the wild-type strain was grown in I⁻ medium and samples were taken from exponential phase through stationary phase. In I⁻ medium, the expression of the *OPI1* gene at each growth phase was greater than the expression observed at the comparable phase of growth for cells grown in I⁺ medium. The peak of expression of the *OPI1* transcript in I⁻ medium appeared to be late exponential phase. Thus, the *OPI1* gene is repressed in the presence of inositol in the growth medium, and is also regulated in a growth-phase-dependent manner (Fig. 7), quite comparable to the pattern seen for the *INO1* transcript in wild-type cells (Fig. 6).

Intracellular inositol content

In both the *opi1* and wild-type strains, maximal levels of intracellular inositol were evident in the samples taken from the early stationary-phase YEPD cultures that were used to inoculate the cultures shown in Fig. 8. The extracts derived from the *opi1* inoculum contained five times the level of inositol detected in the wild-type inoculum (Fig. 8; time point 0). Intracellular inositol content declined during the exponential phase in both strains grown in I⁻ medium. In the *opi1* strain, inositol levels rose again in stationary phase, finally reaching close to 40% of the amount of intracellular inositol that was seen in the starting inoculum. In the wild-type strain, in contrast, intracellular inositol levels remained

Fig. 5. Northern analysis showing the relative abundance of the *INO1* transcript throughout the growth of wild-type (WT-*lacZ*) and *opi1* (OP-*lacZ*) strains in I^- medium (left panels) and I^+ medium (right panels; 100 μ M inositol). Lanes are labelled with the time after inoculation at which the samples were collected. The *TCM1* gene, encoding a ribosomal protein, was included as a control for loading and total RNA synthesis.

Fig. 6. Plot of the abundance of the *INO1* transcript (■, □) expressed as a proportion of total RNA in wild-type (WT-*lacZ*; solid symbols) and *opi1* (OP-*lacZ*; open symbols) strains during growth (●, ○) in I^- medium. Values are the means of at least two determinations.

Fig. 7. Transcriptional expression of the *OPI1* gene. Total RNA was harvested at various points from the wild-type strain (JAGWT) grown in I^+ (100 μ M inositol; ●) and I^- (○) media. Expression was assayed by slot-blot analysis and is displayed as the ratio of the c.p.m. of the *OPI1* signal to the c.p.m. of the *TCM1* signal of the strain as described in Methods. Values are the means of two determinations.

extremely low in the stationary-phase cells growing in I^- medium.

Membrane phospholipid profiles

Membrane PI content was determined in both wild-type and *opi1* mutant strains relative to total membrane phospholipid content. In addition to PI content, the

relative amounts of phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine, phosphatidyltrimethylethanolamine and phosphatidic acid were also quantified. Changes in the proportions of the phospholipids were essentially confined to the four predominant phospholipids, PI, PC, PE and PS, which together accounted for close to 90% of the total 32 P-

Fig. 8. Inositol content of 400 μ l aliquots of intracellular extracts of wild-type (WT-*lacZ*; ●) and *opi1* (OP-*lacZ*; ○) strains during growth in I^- medium. Extracts were prepared and assayed as described in Methods. Values are the means of at least two determinations performed in duplicate.

Fig. 9. Plot of the relative abundance of the four principal phospholipids detected in lipid extracts from wild-type (WT-*lacZ*; a) and *opi1* (OP-*lacZ*; b) strains during growth in I^- medium. Values are the means of three determinations.

labelled lipid material extracted. The relative proportions of PI, PC, PE and PS relative to growth phase are shown in Fig. 9.

The membrane phospholipid composition of both strains was dynamic during the progression through the different growth phases in I^- medium (Fig. 9). In the wild-type strain, the proportion of PI decreased from close to 25% of total phospholipid seen in the inoculum taken from YEPD medium to a value between 5 and 7% of total phospholipid as active growth developed in the absence of inositol. By stationary phase, however, the proportion of PI in the wild-type strain had recovered to

just below 20% of the total phospholipid. Inverse changes occurred in PC content. The relative proportion of PC in the wild-type strain rose to its highest level in the exponential phase and declined slightly as the culture became stationary. Despite the changes observed in PC content in the wild-type strain, this phospholipid remained the most abundant phospholipid species throughout the experiment. The relative proportion of PE changed in a pattern similar to that of PI in the wild-type strain. The proportion of PE was highest in the inoculum and in stationary phase in I^- medium and was lowest during active growth. The least abundant of the major phospholipids in the wild-type inoculum was PS, at a relative proportion of about 10%. It increased slightly to about 15% during the exponential phase and then declined to less than 10% in the stationary phase (Fig. 9).

The relative proportions of the major phospholipids were similar in the *opi1* and wild-type strains in the YEPD-grown cells used for the inoculum (Fig. 9; time point 0). In both strains, the relative proportion of PI declined as the cells entered exponential phase in I^- medium; and in both strains, PC content reached its highest relative levels at this same time. However, at all time points, other than in the starting inoculum which was derived from YEPD medium, relative PI content was higher in the *opi1* strain than in the wild-type. The most striking difference, however, occurred in late stationary phase. The relative proportion of PI in the total phospholipid composition rose higher and the proportion of PC fell lower in the *opi1* strain by late stationary phase (30 h time point; Fig. 9) than in the wild-type strain. At this time point in the *opi1* strain, the relative proportion of PI was actually higher than that of PC. This was the only time point in either the *opi1* or the wild-type strain where the relative proportion of PI exceeded that of PC. A similar inversion occurred in the relative proportions of PE and PS in the *opi1* strain. Again, this was the only time point in either strain where the relative proportion of PS was observed to be greater than PE.

DISCUSSION

This study has revealed for the first time that the *opi1* mutation influences cell growth and morphology, as well as phospholipid metabolism and regulation. The *opi1* mutant strain produces cells of approximately half the diameter of the wild-type strain (Fig. 2) and *opi1* cultures reach a higher cell density at stationary phase than do wild-type cultures (Fig. 1). The *opi1* cells also exhibited a different response than wild-type cells to osmotic shock. Wild-type cells exhibit a rapid but transient decrease in cell diameter (Albertyn *et al.*, 1994; Reed *et al.*, 1987; Rose, 1975) in response to osmotic shock. After a shift from unsupplemented I^- medium to I^- medium supplemented to 8% NaCl, the *opi1* cells were less sensitive to osmotic shock and recovered more quickly from the shift compared with the wild-type cells (Fig. 4). Part of this resistance to the higher salt content of the 8% NaCl medium could be attributable to a

greater resistance to sodium ion toxicity (Watanabe *et al.*, 1995). Smith *et al.* (1996) reported that *cho1* mutants, which lack PS synthase activity, and thus cannot synthesize PC without supplements and display a severe growth disadvantage in rich media, grow as well as the wild-type in high-salt media (0.9 M NaCl). Interestingly, like *opi1* mutants, *cho1* mutants also excrete inositol to produce an Opi⁻ phenotype (Carman & Henry, 1989). These similarities suggest that the overproduction of inositol or some related aspect of phospholipid biosynthesis or regulation may be important for salt tolerance and cell size.

The data presented here also suggest a key role for the *OPI1* gene product in modulating phospholipid metabolism in response to growth phase, as well as in response to the availability of precursors. Previous work on the regulation of phospholipid biosynthesis has focussed primarily on the regulation of key enzymes in response to the availability of the precursors, inositol and choline, during exponential growth (Bailis *et al.*, 1987; Hirsch & Henry, 1986; Klig *et al.*, 1985). It was also reported that the enzymes in yeast, which are subject to regulation in response to inositol and choline, are additionally regulated in response to growth phase (Homann *et al.*, 1987). In wild-type cells, these coregulated enzymes are maximally expressed during exponential growth in the absence of inositol and choline, and are repressed in exponential phase when the precursors are present. However, the enzymes are all expressed at low levels in stationary phase whether the precursors are present or not (Homann *et al.*, 1987). The regulation in response to both precursor availability and growth phase is known to occur at the level of transcript abundance (Bailis *et al.*, 1987; Hirsch & Henry, 1986; Lamping *et al.*, 1995; Paltauf *et al.*, 1992). However, it is not clear how the genetic mechanisms controlling transcription regulation in response to growth phase are related to the controls governing response to soluble precursors.

The transcriptional regulation of the coregulated genes of phospholipid biosynthesis in response to soluble precursors during exponential phase is controlled by at least three *trans*-acting regulatory proteins, the products of the *INO2*, *INO4* and *OPI1* loci. The *INO2* and *INO4* genes encode proteins of the basic helix-loop-helix (bHLH) class (Ambroziak & Henry, 1994; Nikoloff & Henry, 1994). The *INO2* and *INO4* gene products have been shown to form a heterodimer and bind to a repeated sequence known as UAS_{INO} (5'-CATGTGA-AAT-3'), which is found in the promoters of all of the coregulated genes (Paltauf *et al.*, 1992). In the absence of either the *INO2* or the *INO4* gene product, the coregulated genes fail to be derepressed (Hirsch & Henry, 1986; Lopes & Henry, 1991; White *et al.*, 1991). The *OPI1* gene product (Opi1p) is required for repression of all of the coregulated genes of phospholipid biosynthesis, including *INO1* (Hirsch & Henry, 1986; Klig *et al.*, 1985); as shown here in Fig. 5.

In this paper we have demonstrated that the *opi1* mutant is not only defective in its response to soluble precursors

such as inositol, but it also fails to repress the co-regulated genes including *INO1* in stationary phase (Figs 5 and 6). The fact that Opi1p is involved in both aspects of the regulation (i.e. response to phospholipid precursors and growth phase control) suggests that they may both be part of a single mechanism. Alternatively, the exponential-phase and stationary transcriptional responses could involve separate mechanisms; but in that case, the *OPI1* gene product must be required for both. The *OPI1* gene product contains a leucine zipper and polyglutamine stretches, both of which are motifs found in DNA-binding proteins (White *et al.*, 1991). Opi1p has not been demonstrated to bind DNA directly, but it is possible that it exerts its effect indirectly by influencing the binding of the Ino2p-Ino4p complex to UAS_{INO} (Graves, 1996).

Given the role of Opi1p in controlling the levels of transcript abundance of *INO1* and other coregulated genes during both the exponential and stationary phase of growth, we explored the expression of the *OPI1* transcript itself. The expression of the *OPI1* transcript mirrored the pattern of expression of *INO1* (Figs 6 and 7). It is not clear why a gene such as *OPI1*, encoding a regulatory product required for repression, would be expressed at maximal levels under the precise conditions where the genes it controls are also derepressed. However, it may be that Opi1p is required to be present during exponential phase to control the fine tuning of the expression of the coregulated genes, and also it is possible that it must be synthesized in advance of active repression of the coregulated genes.

The absence of the *OPI1* gene product causes major perturbations of both lipid and inositol metabolism in all phases of growth. Cells carrying the *opi1* deletion accumulate larger intracellular pools of inositol, especially in stationary phase. However, for both wild-type and *opi1* cells the highest levels of intracellular inositol were seen in cells transferred from active cultures grown in rich medium (YEPD) (Fig. 8, time point 0). The level of inositol subsequently declined in both strains, with the *opi1* mutants showing higher levels than comparable wild-type cells, no matter what growth condition was compared (Fig. 8).

We found that PI content declines during exponential growth in both wild-type and *opi1* cells, recovering upon entry into the stationary phase. In this way, PI content behaves in a fashion similar to intracellular inositol content. In fact, there is a good correlation ($r = 0.926$) between these two factors using the combined data derived from samples for both strains when cultured in I⁻ medium. This finding is consistent with the observation that PI synthase is not repressed by inositol (Klig *et al.*, 1988) and that the rate of PI synthesis is dictated by the availability of the precursors, inositol and CDP-diacylglycerol (Kelley *et al.*, 1988). The experiments reported here suggest that PI content is largely controlled by inositol availability, relative to growth rate. Relative PI levels continue to rise in the *opi1* mutant even in stationary phase, in concert with

rising intracellular levels of inositol, and this increase appears to be largely at the expense of PC (Fig. 9).

Observations reported here (Figs 8 and 9) suggest that inositol and PI content both become limiting during active growth of yeast cells. This conclusion is supported by the finding that most yeast strains tested grow more rapidly in the presence of inositol than when it is absent, all other growth conditions being equal (Griac *et al.*, 1996). However, the *opi1* mutant differed from the wild-type strain in that its intracellular inositol levels increased during stationary phase in I⁻ medium. In this context, it should be noted that the *opi1* mutants were originally identified on the basis of an inositol excretion phenotype on I⁻ medium on Petri plates (Greenberg & Lopes, 1996). Presumably, the extracellular excretion of inositol is correlated with the increased accumulation of intracellular inositol as the cells enter stationary phase (Fig. 9). Thus, wild-type cells apparently use essentially all of the inositol they make during exponential phase to make phospholipids and they subsequently shut down inositol production in stationary phase by repressing the *INO1* gene. The *opi1* cells, in contrast, fail to repress *INO1* and continue to make inositol even when the cells are not growing. Under these circumstances they cannot utilize all of the inositol produced and the excess accumulates intracellularly and is also excreted, producing the Opi⁻ phenotype seen on Petri plates. These results have important implications for the use of yeast to produce inositol and other phospholipid precursors by yeast cultures.

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