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Inositol and Phosphate Regulate *GIT1* Transcription and Glycerophosphoinositol Incorporation in *Saccharomyces cerevisiae*

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Glycerophosphoinositol is produced through deacylation of the essential phospholipid phosphatidylinositol. In *Saccharomyces cerevisiae*, the glycerophosphoinositol produced is excreted from the cell but is recycled for phosphatidylinositol synthesis when inositol is limiting. To be recycled, glycerophosphoinositol enters the cell through the permease encoded by *GIT1*. The transport of exogenous glycerophosphoinositol through Git1p is sufficiently robust to support the growth of an inositol auxotroph (*ino1*Δ). We now report that *S. cerevisiae* also uses exogenous phosphatidylinositol as an inositol source. Evidence suggests that phosphatidylinositol is deacylated to glycerophosphoinositol extracellularly before being transported across the plasma membrane by Git1p. A genetic screen identified Pho86p, which is required for targeting of the major phosphate transporter (Pho84p) to the plasma membrane, as affecting the utilization of phosphatidylinositol and glycerophosphoinositol. Deletion of *PHO86* in an *ino1*Δ strain resulted in faster growth when either phosphatidylinositol or glycerophosphoinositol was supplied as the sole inositol source. The incorporation of radiolabeled glycerophosphoinositol into an *ino1*Δ *pho86*Δ mutant was higher than that into wild-type, *ino1*Δ, and *pho86*Δ strains. All strains accumulated the most *GIT1* transcript when incubated in media limited for inositol and phosphate in combination. However, the *ino1*Δ *pho86*Δ mutant accumulated approximately threefold more *GIT1* transcript than did the other strains when incubated in inositol-free media containing either high or low concentrations of P_i. Deletion of *PHO4* abolished *GIT1* transcription in a wild-type strain. These results indicate that the transport of glycerophosphoinositol by Git1p is regulated by factors affecting both inositol and phosphate availabilities and suggest a regulatory connection between phosphate metabolism and phospholipid metabolism.

Inositol and phosphate are required for the survival of all eukaryotic cells. In the yeast *Saccharomyces cerevisiae*, each of these nutrients regulates the transcription of a network of genes involved in their metabolism. Starvation for inositol in *S. cerevisiae* results in the coordinated induction of a set of genes involved in phospholipid biosynthesis. The most highly regulated of these is *INO1*, the gene encoding inositol-1-phosphate synthase, the rate-limiting enzyme in inositol biosynthesis (7). *INO1*, as well as the other coregulated genes of phospholipid biosynthesis, contains within its promoter a repeated basic helix-loop-helix (bHLH) consensus sequence (inositol upstream activation sequence [UAS_{INO}]) to which the heterodimerized *INO2* and *INO4* gene products bind to activate transcription when inositol is limiting. Inositol is a precursor of phosphatidylinositol (PI), an essential lipid of eukaryotic cells. PI acts, in turn, as a precursor to sphingolipids, polyphosphoinositides, and glycerophosphoinositol (GroPIns). The GroPIns produced by *S. cerevisiae* is released into the extracellular milieu and represents a major route of PI catabolism in cultures grown in inositol-containing medium (1). When inositol is limiting, GroPIns is transported into the cell, where it is catabolized and its inositol portion is used for the synthesis of PI (19). The transport of GroPIns is mediated through the permease encoded by the *GIT1* gene (20).

Starvation for phosphate (P_i) in *S. cerevisiae* results in the coordinated induction of a set of phosphatase genes (*PHO5*, *PHO10*, and *PHO11*) and the structural gene for low-K_m P_i transport, *PHO84* (11, 17). The phosphoinositol (PHO) system is comprised of five regulatory genes: *PHO2*, *PHO4*, *PHO80*, *PHO81*, and *PHO85*. The induction of *PHO* gene transcription during phosphate starvation is mediated by Pho4p, a bHLH transcription factor, and Pho2p, a homeobox DNA binding protein. The transcriptional status of *PHO5*, a gene whose product supplies more than 90% of the acid phosphatase activity, is typically used as a marker for the entire PHO system. Pho4p binds to two bHLH consensus sequences (CANNTG) in the *PHO5* promoter, and Pho2p binds cooperatively with Pho4p (8). In high-P_i medium, Pho80p and Pho85p form a kinase complex, similar to that formed by a cyclin and a cyclin-dependent protein kinase, which hyperphosphorylates and inhibits Pho4p function, thereby blocking *PHO5* transcription (16). In low-P_i medium, Pho81p inhibits the Pho80p-Pho85p complex and allows the transcription of *PHO5* (25). *PHO5* and the other repressible acid phosphatase (rAPase) genes are constitutively expressed in strains bearing mutations in *PHO84*, the low-K_m P_i transport gene. Other genes thought to be involved in P_i transport include *pho87*, *pho88*, *pho89*, *pho90*, and *pho91* (4, 21, 28). Pho86p is an endoplasmic reticulum protein that is required for the transport of Pho84p to the plasma

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TABLE 1. Strains

Strain	Genotype	Source or reference
JPV1	*trp1 ura3 leu2 his3 MAT*α	P. McGraw
JPV3	*trp1 ura3 leu2 his3 ino1*::*HIS3 MAT*α	P. McGraw
JPV22	*trp1 ura3 thr1 ino1*::*HIS3 MAT***a**	This study
JPV89	*trp1 ura3 leu2 his3 git1*::*HIS3 MAT*α	20
JPV90	*trp1 ura3 thr1 his3 ino1*::*HIS3 pho86*::*URA3 MAT***a**	This study
JPV91	*trp1 ura3 thr1 leu2 his3 MAT***a**	This study
JPV93	*trp1 ura3 thr1 his3 pho86*::*URA3 MAT***a**	This study
JPV95	*trp1 ura3 his3 ino1*::*HIS3 MAT*α	This study
JPV97	*trp1 ura3 his3 git1*::*HIS3 MAT***a**	This study
JPV99	*trp1 ura3 his3 leu2 ino1*::*HIS3 pho86*::*URA3 MAT*α	This study
JPV101	*trp1 ura3 his3 ino1*::*HIS3 git1*::*his3 MAT*α	This study
JPV103	*trp1 ura3 his pho86*::*URA3 git1*::*HIS3 MAT*α	This study
JPV105	*trp1 ura3 his3 thr1 ino1*::*HIS3 pho86*::*URA3 git1*::*HIS3 MAT***a**	This study
JPV139	*trp1 ura3 leu2 his3 ino1*::*HIS3 plb3*::*kanMX4 MAT***a**	This study
JPV142	*trp1 ura3 leu2 his3 ino1*::*HIS3 plb1*::*URA3 plb2*::*kanMX4 MAT***a**	This study
JPV148	*trp1 ura3 leu2 his3 ino1*::*HIS3 plb1*::*URA3 plb2*::*kanMX4 MAT*α	This study
JPV145	*trp1 ura3 leu2 his3 ino1*::*HIS3 MAT***a**	This study
JPV146	*trp1 ura3 his3 leu2 plb1*::*URA3 plb2*::*kanMX4 plb3*::*kanMX4 MAT***a**	This study
JPV155	*trp1 ura3 leu2 his3 ino1*::*HIS3 plb2*::*kanMX4 MAT***a**	This study
JPV163	*ura3 his3 ino1*::*HIS3 plb1*::*URA3 MAT*α	This study
JPV253	*thr1 MAT***a**	S. Henry
MF3	*trp1 ura3 his3 leu2 ade2 plb1*::*URA3 MAT***a**	F. Paltauf
MF11	*trp1 ura3 his3 leu2 ade2 plb2*::*kanMX4 MAT*α	F. Paltauf
MF30	*trp1 ura3 his3 leu2 plb1*::*URA3 plb2*::*kanMX4 plb3*::*kanMX4 MAT***a**	F. Paltauf
JPV203	*ura3 leu2 his3 met15 MAT***a**	Research Genetics
JPV296	*ura3 leu2 his3 lys2 pho4*::*kanMX4 MAT***a**	Research Genetics
JPV339	*ura3 leu2 his3 met15 ino2*::*kanMX4 MAT***a**	Research Genetics

membrane (9). Strains bearing mutations in *PHO86* show constitutive rAPase activity and reduced P_i transport.

The transport of exogenously supplied GroPIns through Git1p supports the growth of an inositol auxotroph (*ino1*Δ), thus defining the Git^+ phenotype (20). We now report that an *ino1*Δ mutant can also grow when PI is supplied as the source of inositol, a phenotype that we have termed Pit^+. Genetic and biochemical studies indicate that *GIT1* transcription and Git1p transport activity are regulated by factors affecting inositol and phosphate availabilities, revealing a metabolic link between phospholipid metabolism and phosphate metabolism.

MATERIALS AND METHODS

Culture conditions. The strains used in this study are listed in Table 1. The strains were grown aerobically at 30°C with shaking. Turbidity was monitored by measurement of the A_{600} with a Beckman DU64 spectrophotometer. Synthetic complete medium was prepared as described previously (19) and contained a high concentration of KH_2PO_4 (7.4 mM). For the experiments represented in Fig. 1 and 2, synthetic complete medium was supplemented with various amounts of inositol, GroPIns (Sigma), or PI (Sigma). High-P_i and low-P_i media (Table 2) (Fig. 3 to 5) were made by replacing KH_2PO_4 (1 g/liter) in synthetic complete medium with KCl (1 g/liter) and adding KH_2PO_4 to 10 mM (high P_i) or 0.2 mM (low P_i). YEPD medium consisted of 20 g of glucose, 10 g of yeast extract, and 20 g of Bacto Peptone per liter.

Strain and plasmid constructions. JPV3 and JPV253 were crossed, and JPV22 was isolated from the resulting diploids by tetrad dissection. JPV90 and JPV89 were crossed, and JPV91, JPV93, JPV95, JPV99, JPV101, JPV103, and JPV105 were isolated from the resulting diploids by tetrad dissection. Mating, sporulation, and tetrad dissection were performed by standard methods (24). Sporulation medium was supplemented with 100 μM inositol. Strains JPV91, JPV93, JPV95, JPV97, JPV99, JPV101, JPV103, and JPV105 were used in the experiments depicted in Table 2 and Fig. 1, 3, and 4. Strains JPV139, JPV142, JPV145, JPV146, JPV155, and JPV163 were used in the experiment represented in Fig. 2. Strains JPV203, JPV296, and JPV339 were used in the experiment represented in Fig. 5.

The bacterial chloramphenicol acetyltransferase (CAT) gene (*cat*) under the control of the *GIT1* promoter was inserted into vector YCp7-32*cat* (13) to produce plasmid pCA998. The following PCR primers (Gibco) were used to amplify the *GIT1* promoter (nucleotides −7 to −881 upstream of the start codon) and to introduce flanking restriction sites (shown in bold type): *Sal*I forward (5′-TCGCCCATGGGG**GTCGAC**TCGATATCTGCGATAAGG-3′) and *Bam*HI reverse (5′-TCGCCCATGGGG**GGATCC**TCCTATTCTATTTTTT T-3′). The PCR product and YCp7-32*cat* were digested with *Bam*HI and *Sal*I and ligated together to form pCA998. To produce a reporter construct with a *LEU2* selectable marker, pCA998 was digested with *Sal*I and *Hin*dIII to release the 2.7-kb fragment in which the *GIT1* promoter is fused to the *cat* gene. The 2.7-kb fragment was ligated to pRS315 (26) to produce pCA999.

Genetic screen for colonies showing fast growth on PI and isolation of *PHO86*. The strain auxotrophic for inositol (JPV3) was transformed to leucine prototrophy with a YEp13-based genomic library. Cells were spread onto plates lacking leucine and incubated at 30°C for 2 days. Colonies were replica plated on synthetic media lacking inositol (I^-), containing 75 μM inositol (I^+), and lacking inositol but containing 75 μM PI. After 3 to 4 days of incubation at 30°C, colonies showing fast growth on plates containing 75 μM PI but unable to grow on I^- plates were selected. Of the 10,000 transformants obtained, 6 displayed increased growth on PI. The plasmids were recovered from yeast cells, amplified in *Escherichia coli*, and retransformed into the parent strain (JPV3) to verify that the plasmids were responsible for the fast growth phenotype. The six plasmids were assorted into two groups based upon restriction fragment mapping. Representative plasmids from each group were sequenced with an ABI Prism 377 automatic DNA sequencer (University of Pittsburgh Research Support Facilities). The open reading frames contained on the complementing plasmids were analyzed by using the *Saccharomyces* Genome Database. One set of complementing plasmids contained the *GIT1* gene. The *GIT1* gene was cloned into multicopy vector pRS424 to produce plasmid pJP104. When transformed with pJP104, strain JPV3 displayed faster growth on PI. The second set of complementing plasmids contained the *PHO86* gene. A 2,177-bp *Sal*I-*Spe*I fragment containing the entire *PHO86* gene was cloned into pRS424 to produce plasmid pJP201. When transformed with pJP201, strain JPV3 displayed faster growth on PI.

Construction of *PHO86* disruption alleles. A 1,641-bp *Hpa*I-*Sph*I fragment containing the entire *PHO86* gene (−568 to 140 bp after the stop codon) was removed from plasmid pJP201 and replaced with a 1,333-bp *Sph*I-*Nar*I fragment containing the *URA3* gene to produce plasmid pJP203. Digestion of pJP203 with *Sal*I and *Spe*I produced a linear fragment containing the *URA3* gene flanked by sequences corresponding to 62 nucleotides at the 5′ end and 474 nucleotides at

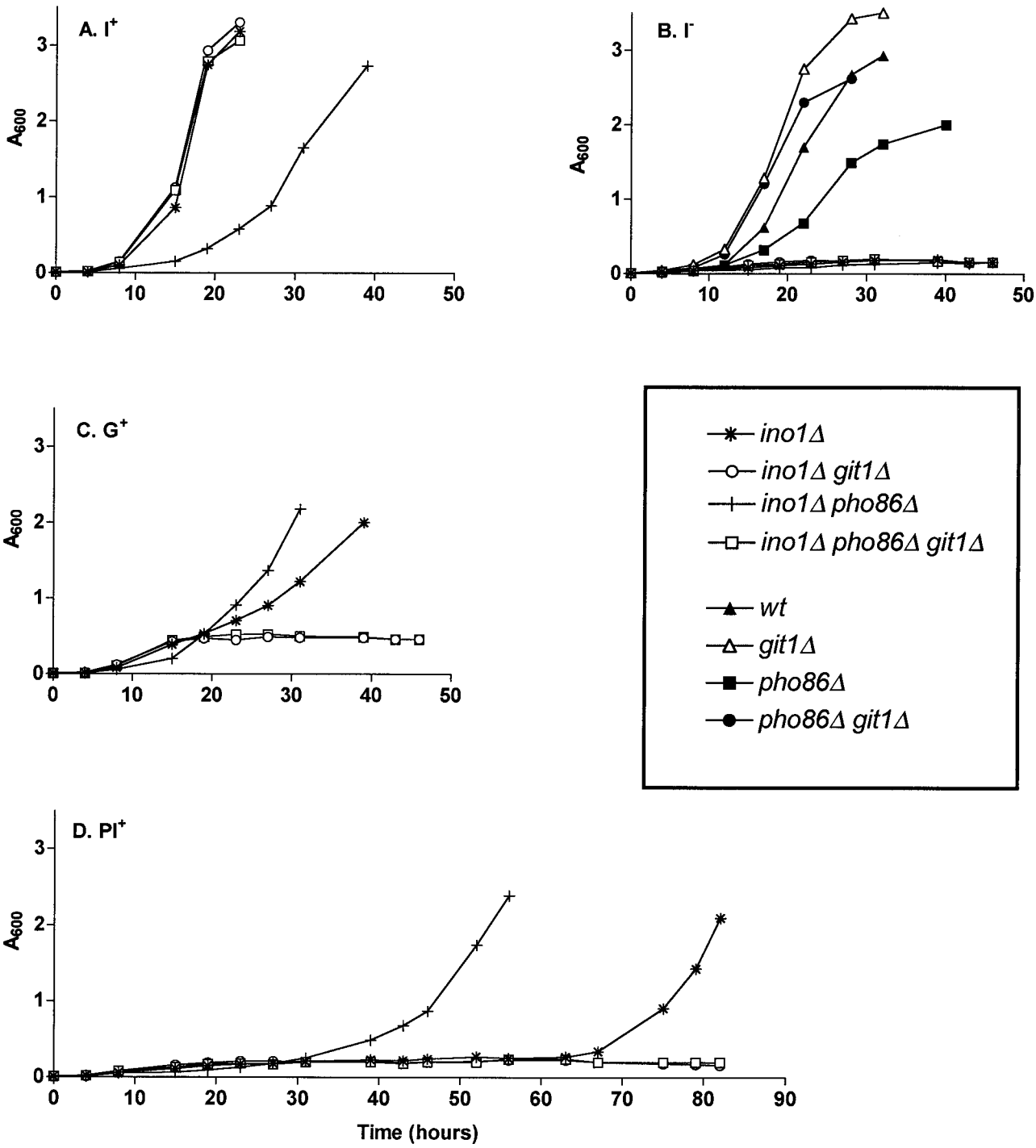

FIG. 1. Deletion of *PHO86* results in enhanced growth of an *ino1*Δ strain in GroPIns-containing and PI-containing media. Strains were grown on synthetic media containing I^+ (A), no inositol source (I^-) (B), 75 mM GroPIns (G^+) (C), and 75 mM PI (PI^+) (D). At the indicated times, the A_{600} was measured. Data for wild-type (wt), *git1*Δ, *pho86*Δ, and *pho86*Δ *git1*Δ strains are shown only in panel B.

the 3′ end of *PHO86*. The *Sal*I-*Spe*I fragment was used for transformation into strain JPV22 by a one-step gene disruption procedure. Uracil prototrophs were screened by PCR to verify integration at the *PHO86* locus, and the resulting strain was named JPV90.

Bacterial and yeast transformations. Bacterial strains were transformed with plasmid DNA by using calcium chloride (24), and yeast strains were transformed by using lithium acetate (24).

^{3}H-GroPIns incorporation. Cell cultures (JPV91, JPV93, JPV95, and JPV99) grown overnight in high-P_i, I^+ synthetic medium were harvested and used to inoculate 5 ml of each of the following media, all of which contained 50 μM glycerophospho-*myo*-[2-^{3}H]inositol (^{3}H-GroPIns) (American Radiolabeled Chemicals Inc.): low P_i, I^-; low P_i, I^+; high P_i, I^-; and high P_i, I^+. After three to five generations, 0.5-ml aliquots of the cultures were centrifuged, and the resulting pellets and supernatants were subjected to liquid scintillation counting

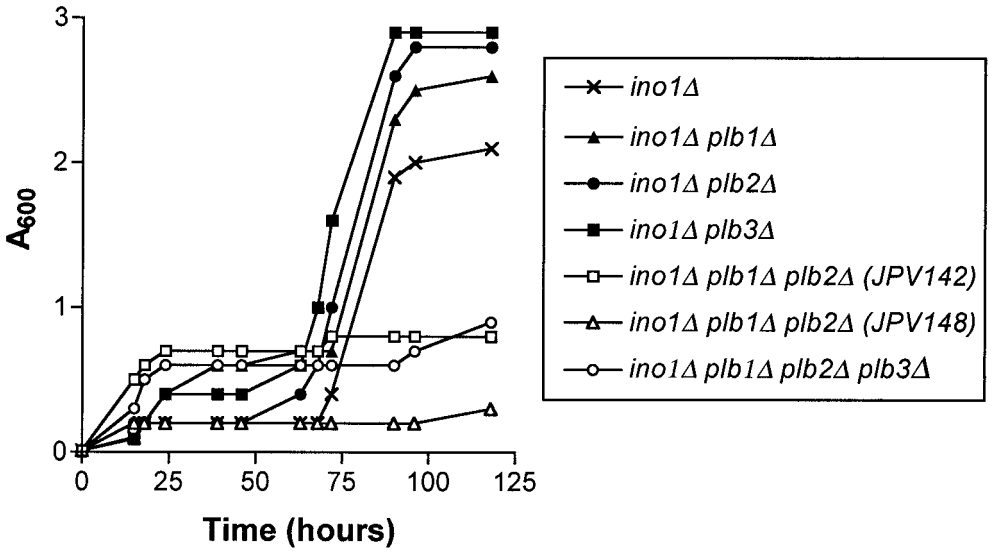

FIG. 2. Growth of an *ino1*Δ mutant on PI requires one or more PLB gene products. Strains were grown on synthetic media containing 75 mM PI, a source of inositol. At the indicated times, the A_{600} was measured.

by using a Beckman LS5801 counter with Ecolume liquid scintillation cocktail (ICN Biomedicals). Strains bearing mutations in the *INO1* gene grew for approximately three generations in I^- media by using their internal reserves of inositol.

Northern analysis. Wild-type cells (JPV91) pregrown in high-P_i, I^+ medium were used to inoculate each of the following media: low P_i, I^-; low P_i, I^+; high P_i, I^-; and high P_i, I^+. Cells were harvested in the logarithmic phase, and RNA was extracted by using the hot acid phenol method (6). RNA was separated on a 1% agarose gel, transferred to a positively charged nylon membrane (Roche catalog no. 1-209-299) by blotting, and UV cross-linked to the membrane by using a Stratalinker. Digoxigenin (DIG)-labeled probes for *GIT1* and control (*SNR17*) transcripts were made by using a Roche PCR DIG probe synthesis kit (catalog no. 1-636-090). Prehybridization, hybridization, and detection were performed in accordance with the manufacturer's instructions for a Roche DIG luminescence detection kit (catalog no. 1-363-514).

CAT assays. Cultures (JPV91, JPV93, JPV95, and JPV99 for Fig. 4; JPV203, JPV296, and JPV339 for Fig. 5) pregrown in high-P_i, I^+ medium were harvested and used to inoculate the following media: low P_i, I^-; low P_i, I^+; high P_i, I^-; and high P_i, I^+. Following three to five generations of growth, lysates were obtained by glass bead breakage (2). The total protein concentration of each lysate was determined with bicinchoninic acid reagent (27). CAT assays were performed in accordance with the manufacturer's protocol for a FAST CAT green (deoxy) CAT assay kit (Molecular Probes catalog no. F-6616). Reactions were carried out for 2.5 h with 5 to 25 μg of protein per assay. Silica gel-coated thin-layer chromatography plates containing the separated reaction products were analyzed by using Kodak Image Station 440.

β-Galactosidase assays. Strains (JPV203, JPV296, and JPV339) were transformed to uracil prototrophy with a plasmid (pJH359) bearing an *INO1-CYC1-lacZ* fusion (12). Cells grown to mid-logarithmic phase in high-P_i, I^+ or in high-Pi, I^- medium were assayed for β-galactosidase activity by using a Pierce Chemical Company yeast β-galactosidase assay kit.

RESULTS

Deletion of *PHO86* accelerates the growth of an *ino1*Δ mutant in GroPIns- and PI-containing medium. *S. cerevisiae* can use exogenous GroPIns as a source of inositol (20). As shown in Fig. 1, *S. cerevisiae* can also use exogenous PI (the precursor of GroPIns) as a source of inositol, albeit with a lag time even greater than that required for growth on GroPIns. In an attempt to identify factors involved in the breakdown and/or utilization of PI and GroPIns by *S. cerevisiae*, a gene overexpression scheme was used. A strain auxotrophic for inositol (*ino1*Δ) was transformed with a high-copy-number YEp13-based genomic library. Transformants displaying an increased

TABLE 2. Incorporation of radiolabeled GroPIns[a]

Expt	Strain	Nmol of GroPIns internalized in medium with the following ingredients:			
		I^-, low P_i	I^+, low P_i	I^-, high P_i	I^+, high P_i
1	Wild type	2.4	2.8	2.9	1.6
	*ino1*Δ	6.1	7.2	4.2	0.6
	*pho86*Δ	4.0	7.9	3.7	1.1
	*pho86*Δ *ino1*Δ	29.4	31.2	21.4	15.5
2	Wild type	29.2	17.6	4.8	2.1

[a] Strains (JPV91, JPV93, JPV95, and JPV99) grown overnight in I^+, high-P_i medium were harvested and inoculated to an A_{600} of 0.05 in 5 ml of each of four separate growth media, each containing 50 μM ^{3}H-GroPIns: low P_i, I^-; low P_i, I^+; high P_i, I^-; and high P_i, I^+. Aliquots of each culture were centrifuged, and the resulting pellets and supernatants were subjected to liquid scintillation counting. For experiment 1, cells were harvested following three or four generations of growth. Data are presented as nanomoles of GroPIns internalized/A_{600} unit. For experiment 2, wild-type cells were harvested at high cell densities (A_{600}, 3 to 4) following 24 h of incubation. The total nanomoles of GroPIns internalized in the stationary phase was determined (data normalized to an A_{600} of 4). Values represent the average of duplicate determinations. These experiments were repeated, with similar results.

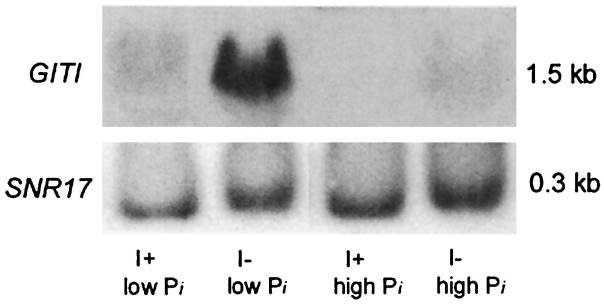

FIG. 3. *GIT1* transcripts accumulate in a wild-type strain limited for phosphate and inositol. A wild-type strain (JPV91) was grown in media containing (I+) or lacking (I−) 75 mM inositol and containing 0.2 mM (low P*i*) or 10 mM (high P*i*) inorganic phosphate. Cells were harvested in the logarithmic phase, and Northern analysis was performed with digoxigenin-labeled probes for *GIT1* and control (*SNR17*) transcripts as described in Materials and Methods.

growth rate when PI was supplied as the inositol source were chosen. Plasmids bearing two different genes were shown to confer the increased growth rate. Those genes were *GIT1* and *PHO86*.

To discern the role of Pho86p in exogenous PI deacylation and GroPIns utilization, *pho86*Δ and *pho86*Δ *ino1*Δ strains were constructed. The *pho86*Δ *ino1*Δ strain grew faster on both GroPIns and PI than the *ino1*Δ strain, and this growth was, in all instances, dependent upon Git1p (Fig. 1C and D). In fact, the *pho86*Δ *ino1*Δ strain (unlike the *ino1*Δ strain) grew at identical rates when supplied with either inositol or GroPIns as an inositol source (compare Fig. 1A and C). The increased growth rate of the *pho86*Δ *ino1*Δ strain on PI was unexpected, since multicopy *PHO86* caused an increased growth rate in the original selection. However, others have reported instances in which overexpression and deletion of the *PHO86* gene result in equivalent phenotypes. Those phenotypes include reduced P_i uptake (4) and the ability to produce rAPase activity under high-P_i conditions (29). Thus, our findings are consistent with other reports and consistent with the notion that both deletion and overexpression of *PHO86* result in a phosphate starvation response. Surprisingly, the *pho86*Δ *ino1*Δ strain grew more slowly than the *ino1*Δ strain when inositol was supplied, but the *pho86*Δ *git1*Δ *ino1*Δ strain grew at the same rate as the *ino1*Δ strain. This result held true outside of an *ino1*Δ genetic background: a *pho86*Δ strain grew more slowly than a wild-type strain, but a *pho86*Δ *git1*Δ strain grew at the same rate as a wild-type strain. These complicated growth phenotypes are a further indication of a functional interaction between Git1p and Pho86p. As expected, strains bearing a deletion in *INO1* were unable to grow in the absence of an inositol source (Fig. 1B). Strains bearing an intact *INO1* gene (*git1*Δ, *pho86*Δ, and *pho86*Δ *git1*Δ) grew similarly in the absence of inositol (Fig. 1B) as in medium containing PI or GroPIns (data not shown).

PI must be deacylated to GroPIns extracellularly to support the growth of an *ino1* mutant. The finding that an *ino1*Δ mutant can grow on exogenous PI only when functional Git1p is present suggested that PI is first deacylated to GroPIns before it is transported into the cell. In order to provide further evidence for this hypothesis, we analyzed strains bearing mutations in the three phospholipase B (PLB) genes that have been characterized for *S. cerevisiae* (10, 14). Plb1p1 and Plb3p are predicted to reside in the plasma membrane and the extracellular space, and Plb2p is predicted to reside in the extracellular space (14). The role of the PLB homologs in exogenous PI deacylation was assessed by monitoring the growth of strains bearing deletions in *INO1* in combination with deletions in the PLB homolog genes when PI was supplied as the

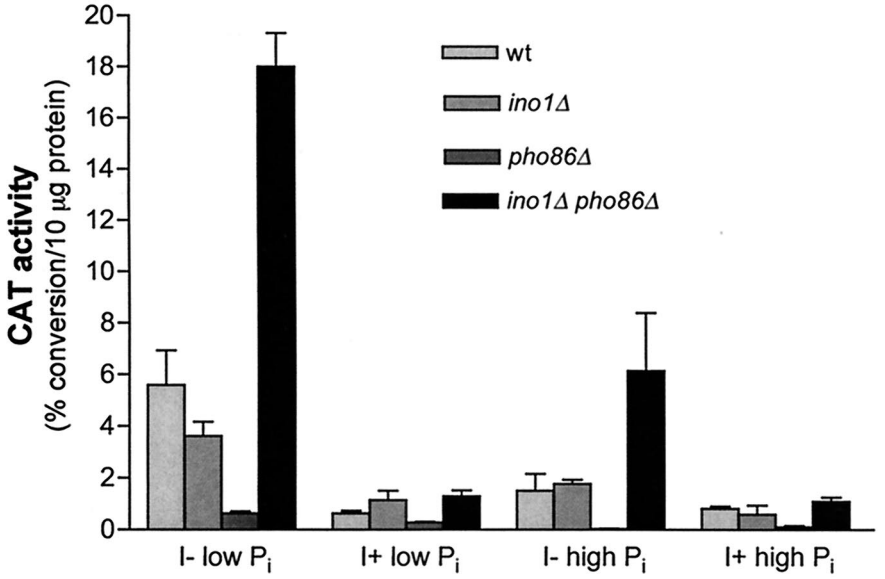

FIG. 4. An *ino1*Δ *pho86*Δ mutant overexpresses *GIT1*, as measured by chloramphenicol acetyltransferase (CAT) activity. Cultures transformed with plasmid pCA998 were grown in high-P_i, I^+ medium lacking tryptophan and were used to inoculate each of the following media lacking tryptophan: low P_i, I^-; low P_i, I^+; high P_i, I^-; and high P_i, I^+. Following three to five generations of growth, lysates obtained by glass bead breakage were assayed for chloramphenicol acetyltransferase activity as described in Materials and Methods. Reactions were carried out for 2.5 h with 5 to 25 μg of protein per assay. Data represent at least three separate determinations and standard errors of the means. wt, wild type.

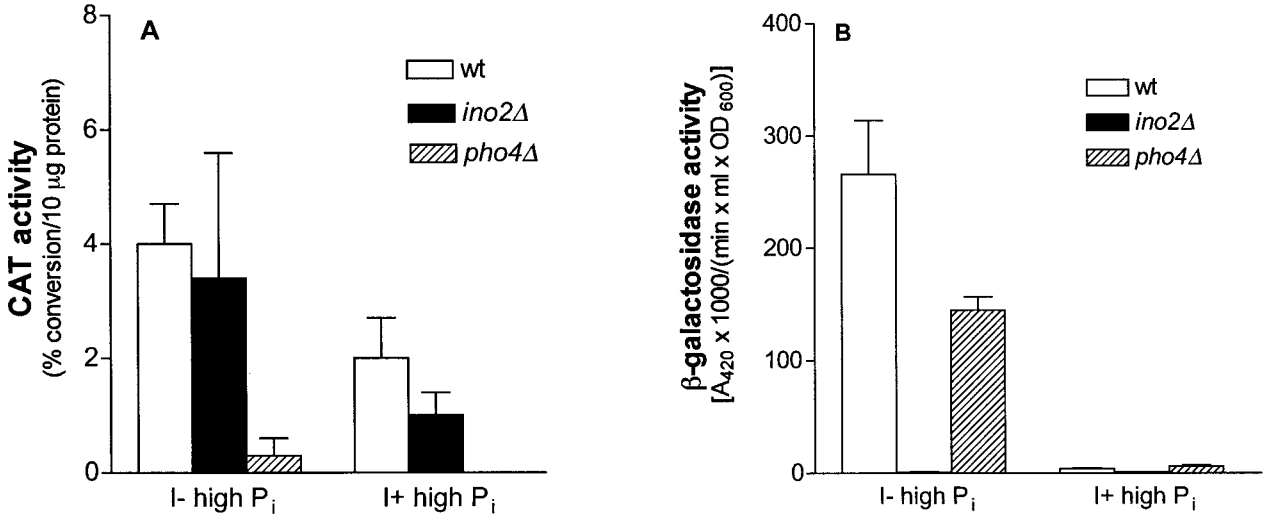

FIG. 5. Pho4p is required for *GIT1* expression but not *INO1* expression. (A) Strains (JPV203, JPV296, and JPV339) transformed with plasmid pCA999 were grown in high-P_i, I^+ medium lacking leucine and were used to inoculate high-P_i, I^- and high-P_i, I^+ media lacking leucine. Following three to five generations of growth, lysates obtained by glass bead breakage were assayed for chloramphenicol acetyltransferase (CAT) activity as described in Materials and Methods. Reactions were carried out for 2.5 h with 5 to 25 μg of protein per assay. (B) Strains (see above) transformed with plasmid pJH359 (*INO1-CYC1-lacZ*) were grown in high-P_i, I^+ medium lacking uracil and were used to inoculate high-P_i, I^- and high-P_i, I^+ media lacking uracil. Following three to five generations of growth, cells were assayed for β-galactosidase activity by using the Pierce yeast β-galactosidase assay kit. Data represent at least three separate determinations and standard errors of the means. wt, wild type; OD_{600}, optical density at 600 nm.

inositol source (Fig. 2). The *ino1*Δ *plb1*Δ, *ino1*Δ *plb2*Δ, and *ino1*Δ *plb3*Δ strains grew at rates similar to that of the *ino1*Δ strain in this experiment. However, two different *ino1*Δ *plb1*Δ *plb2*Δ strains and the *ino1*Δ *plb1*Δ *plb2*Δ *plb3*Δ strain grew more slowly and to a lower density than the *ino1*Δ strain. These results demonstrate that at least one PLB gene product is necessary for the deacylation of exogenous PI to GroPIns.

An *ino1*Δ *pho86*Δ mutant incorporates high levels of exogenous ^{3}H-GroPIns into cells. The increased growth of the *ino1*Δ *pho86*Δ mutant on GroPIns and PI (Fig. 1) indicated that the *PHO86* gene product and, possibly, the phosphate concentration might affect GroPIns transport. To measure ^{3}H-GroPIns incorporation, strains (wild type, *ino1*Δ, *pho86*Δ, and *ino1*Δ *pho86*Δ) pregrown in high-P_i, I^+ medium were used to inoculate four separate media, each containing 50 μM ^{3}H-GroPIns (Table 2): low P_i, I^-; low P_i, I^+; high P_i, I^-; and high P_i, I^+. These experiments were designed to measure ^{3}H-GroPIns transport activity as a function of inositol and phosphate availabilities prior to the utilization of GroPIns as an inositol source. Thus, the cells were harvested and subjected to scintillation counting after three to five generations of growth (under our experimental conditions, strains carrying *ino1*Δ will double approximately three times in I^- medium by using their internal reserves of inositol).

The *ino1*Δ *pho86*Δ strain exhibited greatly increased levels of GroPIns incorporation per cell density compared to the wild-type, *ino1*Δ, and *pho86*Δ strains (Table 2, experiment 1). While the levels of incorporation were not vastly different depending upon the media used, all strains showed the lowest level of incorporation in high-P_i, I^+ medium. When a wild-type strain was grown to a high density (24 h of incubation), the total incorporation of the label varied according to the growth conditions in the following order (highest to lowest): low P_i, I^-; low P_i, I^+; high P_i, I^-; and high P_i, I^+ (Table 2, experiment 2).

***GIT1* expression is sensitive to both inositol and phosphate limitations, and an *ino1*Δ *pho86*Δ mutant overexpresses *GIT1*.** Northern analysis of a wild-type strain indicated that the *GIT1* transcript was most highly expressed under conditions in which both inositol and phosphate were limiting (Fig. 3). A much lower level of *GIT1* expression also occurred in media in which inositol was limiting but phosphate was not and in media in which phosphate was limiting but inositol was not.

In order to facilitate the expression analysis, a plasmid in which the promoter region of *GIT1* was fused to the bacterial *cat* reporter gene in vector yCp7-32*cat* was constructed (13). CAT activity was determined for wild-type, *ino1*Δ, *pho86*Δ, and *ino1*Δ *pho86*Δ strains which were grown initially in high-P_i, I^+ medium and then transferred to four different media which varied in inositol and phosphate concentrations (Fig. 4). In agreement with the results of the Northern analysis, all strains exhibited the most CAT activity when incubated in media limited for both inositol and phosphate. However, an *ino1*Δ *pho86*Δ mutant displayed approximately threefold more CAT activity than the wild-type strain in I^- medium containing both high and low levels of phosphate, suggesting that *GIT1* transcription was hyperinduced.

***GIT1* promoter-driven CAT activity requires Pho4p.** As a first approach to dissecting the regulatory systems involved in controlling *GIT1* transcription, *GIT1* promoter-driven CAT activity was measured for strains bearing deletion mutations in the transcription factors encoded by *PHO4* and *INO2* (Fig. 5A). In high-P_i, I^+ or high-P_i, I^- medium, Pho4p appeared to be required for CAT activity. Under the same growth conditions, strains bearing a deletion in *INO2* displayed decreased

CAT activity. The requirement of Pho4p for *GIT1* transcription was specific, as Pho4p was not required for *INO1* promoter-driven β-galactosidase activity (Fig. 5B). As expected, *INO1* expression required Ino2p (Fig. 5B).

DISCUSSION

S. cerevisiae scavenges nutrients from exogenous GroPIns and PI. While GroPIns enters the cell intact, several lines of evidence indicate that PI must be deacylated to GroPIns prior to being internalized. To begin with, the growth of an *ino1*Δ mutant in PI-containing medium requires *GIT1* (Fig. 1D), and *GIT1* was isolated in the genetic screen as a multicopy suppressor of the slow-growth phenotype of an *ino1*Δ mutant growing in PI-containing medium. Furthermore, the growth of an *ino1*Δ mutant in PI-containing medium requires one or more PLB gene products (Fig. 2); the PLB gene products reside in the extracellular space (14) and hydrolyze phospholipids to produce glycerophosphodiesters, such as GroPIns. Finally, inositol auxotrophic strains that display a Git^- phenotype (*spt7* and *ino2*) (20) also display a Pit^- phenotype (data not shown).

Our prior studies on the regulation of GroPIns transport were performed with media containing high levels of phosphate. Using those conditions, we confirmed that GroPIns transport is regulated by inositol availability (Table 2, compare columns 3 and 4). As an extension of those findings, we now report roles for both inositol and phosphate availabilities in regulating *GIT1* transcription (Fig. 3 and 4) and GroPIns transport (Table 2). In a wild-type strain, the upregulation of *GIT1* transcription in response to inositol limitation is greatly enhanced when phosphate is limiting, just as the upregulation of *GIT1* transcription in response to phosphate limitation is greatly enhanced when inositol is limiting (Fig. 3 and 4). With regard to GroPIns uptake in a wild-type strain, more total ^{3}H-GroPIns is accumulated when cells are grown in low-P_i media as opposed to high-P_i media (Table 2, experiment 2), and that accumulation is increased further by inositol limitation.

Analysis of the *ino1*Δ *pho86*Δ mutant has provided more insight into the regulation of GroPIns utilization. Although the *ino1*Δ *pho86*Δ strain grows slowly in inositol-containing media (Fig. 1A), it displays virtually the same growth rate when grown in GroPIns-containing media (Fig. 1C). This behavior is in stark contrast to that of the *ino1*Δ strain, whose lag phase is greatly lengthened in GroPIns-containing media compared to inositol-containing media (Fig. 1A and C). These results suggest that GroPIns transport and/or catabolism are constitutive in the double mutant. Indeed, GroPIns incorporation is constitutively high, although still regulated by inositol and phosphate, in the *ino1*Δ *pho86*Δ strain (Table 2). Similarly, *GIT1* transcript accumulation in inositol-free media is much higher in the *ino1*Δ *pho86*Δ strain than in any other strain tested. The fact that *GIT1* transcript accumulation in the *ino1*Δ *pho86*Δ strain is similar to that in the wild-type strain grown in I^+ media, while ^{3}H-GroPIns accumulation is much higher, suggests that the regulation of GroPIns accumulation does not occur solely at the level of *GIT1* transcription. Other possible control points affecting GroPIns accumulation include *GIT1* mRNA degradation, Git1p degradation, Git1p transport activity, and the steps involved in the catabolism of GroPIns and its subsequent utilization for de novo PI synthesis. These possibilities are currently under investigation.

Interestingly, both the *ino1*Δ *pho86*Δ (Fig. 1) and the *pho86*Δ (data not shown) strains exhibit a slow-growth phenotype that is alleviated by the deletion of *GIT1*. Strains lacking Pho86p show constitutive rAPase activity and are unable to efficiently transport Pho84p to the plasma membrane (9). Similarly, *pho84*Δ mutants show constitutive rAPase activity and have no Pho84p. As an initial attempt to dissect these complex phenotypes, we will examine whether the deletion of *GIT1* in a *pho84*Δ mutant (and in strains carrying mutations in other *PHO* genes) affects growth, as it does in a *pho86*Δ background.

In terms of GroPIns incorporation and *GIT1* transcript accumulation, the *ino1*Δ *pho86*Δ mutant behaves in a manner much different from that of either the *ino1*Δ or the *pho86*Δ mutant alone and different from that of the wild-type strain grown in media limited for inositol and phosphate. This finding suggests a synergistic role for inositol and phosphate in regulating the utilization of GroPIns. Furthermore, it suggests that the cell is sensitive to the severity of inositol and phosphate limitation. A wild-type strain growing in I^-, low-P_i medium is still capable of making its own inositol and transporting P_i into the cell. An *ino1*Δ *pho86*Δ mutant growing in I^-, low-P_i medium, on the other hand, has no source of inositol (but grows for a limited time on its internal reserves), has a greatly diminished ability to transport P_i, and shows hyperactivated *GIT1* transcription and GroPIns incorporation.

Using genome-wide expression analysis, others have reported that the *GIT1* transcript accumulates when phosphate limitation is artificially imposed by deletion of *PHO85* or chemical inhibition of Pho85p (5). In another microarray study, *GIT1* did not meet the authors' criteria for being a phosphate-regulated gene but did display the induction of transcription in low-P_i versus high-P_i media in one of two wild-type strains tested (15). Those studies were performed with I^+ media. In wild-type strain JPV91, *GIT1* transcription is significantly affected only by phosphate concentrations in I^- media. Thus, the relative contributions of inositol depletion and phosphate depletion in affecting *GIT1* transcription appear to be strain dependent. Wykoff and O'Shea (28) reported that the overexpression of *GIT1* in high-P_i medium suppresses the nonviability of a strain from which all other known phosphate transporter genes (*PHO84*, *PHO87*, *PHO89*, *PHO90*, and *PHO91*) have been deleted. The authors concluded that Git1p is capable of transporting P_i, albeit with a higher K_m for P_i than that exhibited by Pho84p. Although differences in strain backgrounds and assay conditions preclude a direct comparison, it is worth noting that the reported apparent K_m for P_i transport by Git1p (28) is approximately 10-fold higher than the reported apparent K_m for GroPIns transport by Git1p (19).

An initial experiment (Fig. 5) aimed at determining the regulatory systems responsible for controlling *GIT1* expression indicates that *PHO4* is required for *GIT1* transcription under high-phosphate conditions. Future studies will include a thorough analysis of all potential transcription factors (e.g., Ino4p, Ino2p, Pho4p, and Pho2p) and promoter elements involved in controlling *GIT1* expression in response to inositol and phosphate. Pho4p, the PHO regulon transcription factor, is a bHLH binding protein whose consensus sequence is CACGTK

(17). Five potential Pho4p binding sites and three potential Pho2p binding sites (TAATRA/TAANTAA) exist in the *GIT1* promoter region between nucleotides −1 and −700 relative to the ATG start codon. The *GIT1* promoter does not contain a copy of the core consensus sequence (CATGTG) for UAS_{INO}. However, it does contain a bHLH consensus sequence (CAC GTG) to which Ino2p and Ino4p (bHLH proteins) bind, although with less affinity than to UAS_{INO}, to activate *INO1* transcription (3). Given that mammalian bHLH binding proteins have been shown to form multiple dimer combinations that can act upon diverse sets of genes (23), it is tempting to speculate that Pho4p may heterodimerize with Ino2p or Ino4p to regulate *GIT1* transcription in response to inositol and phosphate. In support of this possibility, Ino4p has been shown by a yeast two-hybrid assay and a biochemical assay to interact with Pho4p, as well as other bHLH binding proteins (22). Interestingly, *PHO5* expression is partially repressed by inositol availability and the deletion of *PHO2* (18). In addition to promoter analysis, future studies will include an analysis of *GIT1* mRNA turnover and Git1p turnover as functions of inositol and phosphate availabilities.

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