Glycerophosphoinositol, a Novel Phosphate Source Whose Transport Is Regulated by Multiple Factors in Saccharomyces cerevisiae*

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Git1p mediates the transport of the phospholipid metabolite, glycerophosphoinositol, into Saccharomyces cerevisiae. We report that phosphate limitation and inositol limitation affect GIT1 expression and Git1p transport activity via distinct mechanisms that involve multiple transcription factors. GIT1 transcript levels and Git1p activity are greater in cells starved for phosphate, with or without inositol limitation, than in cells only limited for inositol. Furthermore, the kinetics of GIT1 transcript accumulation and Git1p activity upon transfer of cells to phosphate starvation media are different from those obtained upon transfer of cells to inositol-free media. Pho2p and Pho4p are required for GIT1 expression and for Git1p transport activity under all growth conditions tested. In contrast, Ino2p and Ino4p are required for full GIT1 expression when inositol is limiting, with or without phosphate limitation, but not when only phosphate is limiting. Greatly reduced transport activity was detected in ino2Δ and ino4Δ cells under all growth conditions. A 300-base pair region of the GIT1 promoter containing potential Pho4 binding sites was shown to be required for full GIT1 expression. Git1p appears to act as a H⁺-symporter, and neither inositol nor phosphate effectively compete with glycerophosphoinositol for transport by Git1p. Glycerophosphoinositol was shown previously to support the growth of an inositol auxotroph. Remarkably, we now report that glycerophosphoinositol can act as the sole source of phosphate for the cell, providing functional relevance for the regulation of Git1p transport activity by phosphate.

Cells must integrate the multiple signals that they receive from their environment and adjust their metabolism accordingly. Because phosphate is a required nutrient, organisms have evolved ways of sensing extracellular phosphate, scavenging it from the environment, and transporting it into the cell. In Saccharomyces cerevisiae, phosphate starvation induces the transcription of phosphatase genes (PHO5, PHO10, PHO11), the structural gene for low Kₚ transport, PHO84, and several other genes that have been identified through traditional and microarray approaches (1–5). Five regulatory genes control the PHO regulon, PHO2, PHO4, PHO80, PHO81, and PHO85. Pho4p, a basic helix-loop-helix (bHLH)² transcription factor, and Pho2p, a homeobox DNA-binding protein mediate the induction of PHO transcription during phosphate starvation. In a high Pₗ medium, Pho80p and Pho85p form a kinase complex similar to that formed by a cyclin and a cyclin-dependent protein kinase, which phosphorylates Pho4p. Phosphorylated Pho4p is concentrated in the cytoplasm where it cannot activate transcription (6). In low Pₗ medium, Pho81p inhibits the Pho80p-Pho85p complex, blocking phosphorylation of Pho4p. Dephosphorylated Pho4p is concentrated in the nucleus where it can activate transcription (6).

Growth of S. cerevisiae in inositol-free medium results in the coordinate 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). Cells starved for exogenous inositol, therefore, synthesize inositol de novo. INO1, as well as the other co-regulated genes of phospholipid biosynthesis, contains within its promoter a repeated bHLH consensus sequence (UASINO) to which the heterodimerized INO2 and INO4 gene products bind to activate transcription when inositol is limiting (8, 9). Inositol is a precursor of phosphatidylinositol (PI), an essential lipid of eukaryotic cells. PI acts, in turn, as a precursor to sphingolipids (IPC, MIPC, M(IP)₂C and polyphosphoinositides (PIP, PIP₂, PIP₃). PI deacylation via phospholipases B (encoded by PLB1, PLB2, and PLB3) results in the production of glycerophosphoinositol (GroPIns). GroPIns is released into the extracellular milieu and is a major PI catabolite in cultures grown in inositol-containing medium (10). 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 (Fig. 1) (11). Transport of GroPIns is mediated through the permease encoded by the GIT1 gene (12). The predicted GIT1 protein product bears similarity to other S. cerevisiae transport proteins, including the inositol transporters Inr1p and Inr2p, and the inorganic phosphate transporter, Pho84p. Recent studies (13) have shown that in addition to inositol limitation, phosphate limitation induces increased uptake of GroPIns. In the present report, we characterize further the nature of the regulation of GroPIns transport via inositol and phosphate limitation. Regulation of GIT1 expression is shown to occur at multiple levels and to be multifactorial. Importantly, we report that GroPIns, when supplied as the sole phosphate source, supports the robust growth of S. cerevisiae in a GIT1-dependent manner.

EXPERIMENTAL PROCEDURES

Strains and Media—Strains (Table I) were grown aerobically at 30 °C with shaking. Turbidity was monitored by measurement at A₆₀₀ nm on a Biometra 3 Thermo Spectronic spectrophotometer. Synthetic complete medium was prepared as described previously (11). “High Pₗ,” “Low Pₗ,” and “no Pₗ” media, (see Table II and Figs. 3, 4, and 5) were made by replacing the KH₂PO₄ (1 g/liter) in synthetic complete medium

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1 The abbreviations used are: bHLH, basic helix-loop-helix; GroPIns, glycerophosphoinositol; PI, phosphatidylinositol; CAT, chloramphenicol acetyltransferase.
Glycerophosphoinositol Transport in Yeast

Table I

| Yeast strains |
|---------------|
| Strain | Genotype | Source |
| Wild type (JPV203) | his3Δ1, leu2Δ2α, met15α, ura3Δ0, MATα | Research Genetics |
| G111-HA (JPV373) | his3Δ1, leu2Δ2α, met15α, ura3Δ0, G111-HA, MATα | This study |
| ino2Δ (JPV339) | his3Δ1, leu2Δ2α, met15α, ura3Δ0, ino2::KanMX, MATα | Research Genetics |
| ino4Δ (JPV340) | his3Δ1, leu2Δ2α, met15α, ura3Δ0, ino4::KanMX, MATα | Research Genetics |
| pho2Δ (JPV296) | his3Δ1, leu2Δ2α, met15α, ura3Δ0, pho2::KanMX, MATα | Research Genetics |
| pho4Δ (JPV296) | his3Δ1, leu2Δ2α, met15α, ura3Δ0, pho4::KanMX, MATα | Research Genetics |
| git1Δ (JPV212) | his3Δ1, leu2Δ2α, met15α, ura3Δ0, git1Δ::KanMX, MATα | Research Genetics |

**Fig. 1. Glycerophosphoinositol metabolism in S. cerevisiae.** GroPIns produced through PI deacetylation exit the yeast cell via an unknown mechanism. GroPIns is transported into the cell via the G111 gene product. S. cerevisiae is capable of deacetylating exogenous PI to produce GroPIns. Inside the cell, GroPIns is catabolized, and the inositol portion is used for the synthesis of PI. The gene products involved in GroPIns catabolism have not been delineated. Gly-3-P, glycerol-3-phosphate; Gly, glycerol; Ins, inositol. Genes are denoted by italics.

**Northern Analysis**—Wild type, pho2Δ, and pho4Δ strains were grown to log-phase in each of the following media: 1) low P, I−; 2) high P, I−; 3) high P, I+. For the inositol auxotrophs, ino2Δ and ino4Δ, I− media were substituted for I− media. RNA was extracted, and Northern blots were performed as described (13).

**Chloramphenicol Acetyltransferase Assays**—Strains (wild type, pho2Δ, pho4Δ, ino2Δ, and ino4Δ) pregrown in high P, I− medium were harvested and used to inoculate the following media: 1) low P, I−; 2) high P, I−; 3) high P, I−; 4) high P, I+. For the auxotrophs, ino2Δ and ino4Δ, I− media were substituted for I− media. At the indicated times, aliquots of the cultures were harvested, and CAT activity was determined using the FAST CAT green (deoxy) chloramphenicol acetyltransferase assay kit as described (13).

**Production of Radiolabeled GroPIns**—Tritium-labeled GroPIns (1[H]GroPIns) was produced through the deacetylation of phosphatidylmyo-1-2-H]-inositol (American Radiolabeled Chemicals) as described (14).

**GroPIns Transport Assays**—Strains (wild type, pho2Δ, pho4Δ, ino2Δ, and ino4Δ) pregrown in high P, I− medium were harvested and used to inoculate the following media: 1) low P, I−; 2) low P, I−; 3) high P, I−; 4) high P, I+. For the auxotrophs, ino2Δ and ino4Δ, I− medium was substituted for I− medium. At the indicated times, aliquots of the cultures were harvested and washed once with sterile water. The cell pellet was suspended in 100 mM potassium citrate buffer, pH 4.0, to a density of 5 000 cells/mL. The reaction was started by the addition of 10 μl of 25 μM [1[H]GroPIns to 200 μl of the cell suspension. Following a 5-min incubation at 30 °C, the reaction was stopped by the addition of 10 ml of ice-cold H2O. The samples were filtered through glass fiber (GF/C) filters, and the filters were washed with 15 ml of ice-cold H2O. Radioactivity on the filter was determined by liquid scintillation counting using a 2100TR Packard Liquid Scintillation Analyzer.

**Construction of G111-cat Fusion Plasmids**—For the construction of plasmid pWC639, a 2.4-kb fragment containing a portion of the G111 promoter (nucleotides from −7 to −689 upstream of the start codon) fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (cat) was amplified by PCR using pCA999 as a template. Primers contained homology to pCA999 as well as sequences to introduce flanking restriction sites for Sall and HindIII and ligated to produce pWC639. To produce pWC335, a 2.05-kb fragment (containing nucleotides from −7 to −355 upstream of the start codon) was amplified by PCR using pCA999 as a template. Primers contained homology to pCA999 as well as sequences to introduce flanking restriction sites for Sall and HindIII and ligated to produce pWC335.

**Construction of Chromosomal G111-HA Allele**—The plasmid pFA6-a-3HA-kanMX6 (15) kindly provided by M. Longtine (Oklahoma State University, Stillwater, OK) was used as template to amplify a 3′HA-kanMX module for insertion into the genome at the 3′-end of G111. The 5′-ends of the forward and reverse primers bore 40 nucleotides homologous to the target gene sequences, followed by 20 nucleotides homologous to the 3′-HA-kanMX module, forward primer 5′-AACGAAACGAGGAGAAAGAGCCCGCCGAAAGGGATCCATGTTTGACGACGAGCTTGAGACCGAGCTTGAAGAAAACGC-3′ and HindIII (reverse primer, 5′-TTTGCTGGATCCATGTTAGATCTTGACGACGAGCTTGAGACCGAGCTTGAAGAAAACGC-3′). The PCR product and plasmid pRS315 were digested with Sall and HindIII and ligated to produce pWC639. To produce pWC335, a 2.05-kb fragment (containing nucleotides from −7 to −355 upstream of the start codon) was amplified by PCR using pCA999 as a template. Primers contained homology to pCA999 as well as sequences to introduce flanking restriction sites for Sall and HindIII and ligated to produce pWC335.

**Western Blot Analysis**—Wild type cells grown in 20 ml of the indicated media were collected in late logarithmic phase, suspended in 200 μl of ice-cold extraction buffer (120 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.1% SDS, and 1% Triton X-100), and lysed with glass beads (18). Unbroken cells and debris were removed by centrifugation, and protein concentrations were determined using the bicinchoninic acid protein assay kit (Sigma). Samples were heated at 37 °C for 10 min with Tris-Gly SDS sample buffer (Novex). A total of 40–50 μg were loaded/ lane into 4–20% Tris-Gly gels (Invitrogen). Gels were transferred to an Invitronol polyvinylidene difluoride membrane (Invitrogen) using the Xcell II Blot Module (Invitrogen). Membranes were stained with Pon-
ceau S 0.5% in 1% acetic acid to check for efficiency of transfer. Membranes were blocked in phosphate-buffered saline, 20% Tween 20 containing 1% bovine serum albumin overnight at 4 °C. Membranes were equilibrated for 15 min at room temperature with shaking. Primary antibody (monoclonal antibody HA-11, Covance catalog number MMS-101P) was added to solution at 1:5000 dilution and incubated at room temperature for 1 h. Primary antibody solution was removed, and membranes were washed with phosphate-buffered saline, 20% Tween 20 for 30 min, changing the solution every 5–6 min. Membranes were incubated with secondary antibody (peroxidase-labeled affinity-purified antibody to mouse IgG (H+L), KPL catalog number 074-1806) in 1% bovine serum albumin/phosphate-buffered saline, 20% Tween 20 at 1:75000 dilution for 1 h at room temperature. The membrane was washed as described above, incubated with SuperSignal West Dura extended duration substrate (Pierce) for 5 min at room temperature before exposing to Kodak Biomax MR Film for visualization.

RESULTS

Multiple Transcription Factors Affect GIT1 Transcript Abundance—The roles of the transcription factors Ino2p, Ino4p, Pho2p, and Pho4p in regulating GIT1 transcription in response to inositol and phosphate availability were investigated. Wild type cells grown to logarithmic phase in \( I^- \) and low Pi media accumulated a GIT1 transcript (Fig. 2). When cells were grown in high Pi conditions, no transcript was detected. The \( \text{pho}4^- \) and \( \text{pho}2^- \) mutants accumulated no detectable GIT1 transcript under any growth conditions. Interestingly, \( \text{ino}2^- \) and \( \text{ino}4^- \) mutants accumulated no GIT1 transcript in

![GIT1 probe](image1)

![SNR17 probe](image2)

**Fig. 2.** Multiple transcription factors affect GIT1 transcript abundance. Media lacked inositol (\( I^- \)) or contained 75 \( \mu \)M inositol (\( I^+ \)) and contained 0.2 mM (L) or 10 mM (H) KH\(_2\)PO\(_4\). For \( \text{ino}2^- \) and \( \text{ino}4^- \) strains, media indicated as \( I^- \) contained 5 \( \mu \)M inositol. RNA extraction and Northern blot analyses were performed as described under “Experimental Procedures.” Hybridization with the SNR17 probe served as a loading control. Data are representative of two independent experiments.

![A. Wild type](image3)

![B. ino4^-](image4)

![C. pho4^-](image5)

**Fig. 3.** GIT1 promoter activity does not necessarily correlate to Git1p transport activity. Strains grown in medium containing 75 \( \mu \)M inositol and 10 mM KH\(_2\)PO\(_4\) were harvested and reinoculated into medium lacking inositol (\( I^- \)) or containing 75 \( \mu \)M inositol (\( I^+ \)), with either 0.2 mM (L) or 10 mM (H) KH\(_2\)PO\(_4\). For \( \text{ino}2^- \) and \( \text{ino}4^- \) strains, media indicated as \( I^- \) contained 5 \( \mu \)M inositol. GIT1-promoter driven CAT activity, GroPIns transport activity, and growth were measured as described under “Experimental Procedures.” Values represent mean ± S.E. of triplicate determinations.
Inositol and phosphate availability affect GIT1-HA protein abundance. Strain GIT1-HA was grown to logarithmic phase in each of the indicated media. Immunoblots were performed as described under “Experimental Procedures.”

**FIG. 4.** Inositol and phosphate availability affect GIT1-HA protein abundance. Strain GIT1-HA was grown to logarithmic phase in each of the indicated media. Immunoblots were performed as described under “Experimental Procedures.”

**TABLE II**

Effect of compounds on GroPIns transport

| Addition                  | Activity %  |
|---------------------------|-------------|
| None                      | 100         |
| CCCP (50 μM)              | None detected |
| Inositol (1 mM)           | 129 ± 3     |
| Sodium phosphate (1 mM)   | 180 ± 15    |
| GroPIns (1 mM)            | 15 ± 1      |

Effect of compounds on GroPIns transport. Transport activity was assayed in the presence of 100 mM sodium citrate buffer, pH 4.0, 5 μM glycerophosphoinositol, and the indicated concentrations of added compounds. Values are the percentage of activity remaining as compared to the activity obtained when no additions were made (none). Values represent the mean ± S.E. of triplicate determinations.

Deletion of Potential Pho4p Binding Sites Results in Loss of GIT1 Promoter Activity—To identify the regions of the promoter important for GIT1 expression, a promoter deletion analysis was performed (Fig. 5). GIT1 promoter-driven CAT activity was compared in cells harboring one of three plasmids, plasmid pCA999 contains nucleotides from the start codon, plasmid pWC639 contains nucleotides from the start codon, and plasmid pWC335 contains nucleotides from the start codon to the phosphatase transfer of wild type cells into the I−, low Pi condition but accumulated wild type levels of transport (12, 13), prompted us to perform a more in-depth analysis of GIT1 expression and its relationship to transport activity. GIT1 expression was monitored via a reporter construct in which the GIT1 promoter was fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (cat) (13). GIT1 promoter activity as well as GroPIns transport activity were monitored as a function of time following the transfer of cells from fully repressing medium (I+, high Pi) to various starvation media (Fig. 3).

In contrast to the results obtained by Northern analysis, transfer of wild type cells into the I−, high Pi, medium did result in a modest, but clearly detectable, increase in CAT activity. The transfer of wild-type cells into low phosphate media (either containing or lacking inositol) resulted in a much larger increase in CAT activity that continued to rise even as the cells entered the postdiauxic shift (growth data not shown).

The transport activity profiles looked quite different from those obtained for CAT activity. When wild-type cells were grown under low phosphate conditions (with or without inositol), transport activity reached a maximum at 12 h and then declined. In contrast, cells starved for inositol alone displayed a smaller but sustained increase in transport activity (Fig. 3A).

We have reported previously (12) that GroPIns transport activity is entirely GIT1-dependent at the concentration used in the assay (5 μM). In addition, no transport was detected when a git1Δ strain was used as a control in the experiments presented here (data not shown).

The ino4Δ mutant grown in the I+, low Pi condition exhibited CAT activity similar to wild-type cells grown in the same conditions (Fig. 3B). In contrast, when ino4Δ cells were grown in the I−, low Pi condition, no CAT activity was detected. Interestingly, ino4Δ cells displayed greatly reduced transport activity as compared with wild-type cells under all growth conditions tested. The ino2Δ strain behaved nearly identical to the ino4Δ in terms of both CAT activity and transport (data not shown). These results suggest that Ino2p and Ino4p are able to affect transport in a manner independent of their role in regulating GIT1 transcription. Strains bearing deletions in PHO4 (Fig. 3C) or PHO2 (data not shown) produced little or no GIT1 promoter-driven CAT activity or GroPIns transport activity under any growth condition.

Deletion and Phosphate Availability Affect Git1p Abundance—Wild-type cells bearing a chromosomal GIT1-HA construct were grown in I+. High Pi, medium was transferred to the three growth conditions in which transport activity was detected, I−, low Pi; I−, low Pi; and I−, high Pi. Similar to the transport profiles, Git1p levels peaked and then declined as a function of time in cells starved for phosphate (Fig. 4). Little Git1p was detected in cells grown in I−, high Pi, medium, consistent with the relatively low level of transport activity present under those conditions (Fig. 3A).

Inositol and Phosphate Are Not High Affinity Substrates for Git1p—To determine whether inositol and/or phosphate directly affect transport activity, competition experiments were performed (Table II). If a compound has affinity for the transporter, we would expect that the addition of that compound (nonradioactive) to the assay mixture would compete with ra-
diolabeled GroPIns and decrease the apparent transport activity. As expected, the addition of 200-fold excess of unlabeled GroPIns caused a large reduction in transport activity. In contrast, transport of radiolabeled GroPIns was unaffected by 200-fold excess of unlabeled inositol and was actually stimulated by a 200-fold excess of unlabeled sodium phosphate. Therefore, inositol and phosphate do not compete with GroPIns for transport by the Git1p permease. The results obtained by way of optimizing the GroPIns transport assay support the prediction that Git1p acts as a H$^+$ symporter (20). Transport activity decreases as the pH of the assay buffer increases (Fig. 6). Furthermore, the protonophore carbonyl cyanamide m-chlorophenylhydrazone, which destroys proton gradients, completely abolished transport activity (Table II).

\textbf{S. cerevisiae Can Utilize GroPIns as Sole Phosphate Source—}

The increase in \textit{GIT1} transcript and GroPIns transport activity by phosphate starvation suggested that yeast may utilize GroPIns as a source of phosphate. To test this hypothesis, cells grown in low phosphate medium were harvested, washed, and spotted onto phosphate-free culture plates lacking a phosphate source containing 75 $\mu$M KH$_2$PO$_4$ or containing 75 $\mu$M GroPIns. Wild type cells grew when provided with GroPIns as their sole source of phosphate in a \textit{GIT1}-dependent fashion (Fig. 7). In contrast, the \textit{pho2}Δ and \textit{pho4}Δ mutants could not utilize GroPIns as the sole phosphate source. Interestingly, the \textit{ino2}Δ and \textit{ino4}Δ mutants grew similarly to wild type in both I$^+$, GroPIns medium and I$^-$, GroPIns medium. When the inositol auxotrophs \textit{ino2}Δ and \textit{ino4}Δ are grown in I$^-$, GroPIns medium, GroPIns is acting as the sole phosphate and sole inositol source (note that \textit{ino2}Δ and \textit{ino4}Δ could grow in I$^+$ medium containing 75 $\mu$M KH$_2$PO$_4$ but could not grow in I$^-$ medium containing 75 $\mu$M KH$_2$PO$_4$).

\textbf{Discussion}

Our results indicate that the signals of phosphate limitation and inositol limitation induce different but overlapping regulatory programs with regard to \textit{GIT1} expression. For example, the induction of \textit{GIT1} transcript accumulation upon phosphate limitation does not require \textit{INO2} and \textit{INO4}, but \textit{INO2} and \textit{INO4} are required when phosphate limitation is combined with inositol limitation (Figs. 2 and 3). \textit{PHO2} and \textit{PHO4}, on the other hand, are required for \textit{GIT1} transcript accumulation under all growth conditions tested (Figs. 2 and 3).

The magnitude of the response elicited by phosphate starvation is much larger than that caused by inositol limitation in terms of expression and transport activity. We detected abundant \textit{GIT1} transcript (Fig. 2) and \textit{GIT1} promoter-driven CAT activity (Fig. 3) in cells grown in low P$_i$, media with or without inositol. In contrast, we detected no transcript in cells grown in I$^-$, high P$_i$, medium (Fig. 2) and detected low but reproducible levels of \textit{GIT1} promoter-driven CAT activity (Fig. 3). A possible explanation for the discrepancy between \textit{GIT1} message and promoter activity in I$^-$, high P$_i$ conditions is that the \textit{GIT1} message is very unstable, whereas the heterologous \textit{GIT1}-cat message is less so. Transport activity is also differentially affected by phosphate limitation as compared with inositol limitation. Wild type cells grown in low P$_i$, medium with or without inositol display severalfold greater transport activity than those grown in I$^-$, high P$_i$ medium. Our inability to detect GroPIns transport in \textit{git1}Δ delete strains indicates that Git1p is indeed responsible for the GroPIns transport detected not an alternate transporter whose transcript we did not monitor.

Our results suggest that post-transcriptional mechanisms are also likely to be involved in the regulation of Git1p activity. When \textit{ino2}Δ and \textit{ino4}Δ are transferred to I$^+$, low P$_i$, medium, they exhibit an induction of CAT activity similar to that seen in the wild type strain but maximal transport activity that is roughly 4-fold less than that of the wild type strain (Fig. 3). Thus, \textit{Ino2p} and \textit{Ino4p} appear to affect, directly or indirectly, Git1p activity via a post-transcriptional mechanism in cells grown in I$^+$, high P$_i$ medium. One possibility is that \textit{Ino2p} and \textit{Ino4p} are required for the transcription of an unknown gene whose product activates Git1p. The exact nature of the post-transcriptional control rendered by \textit{Ino2p} and \textit{Ino4p} will be the subject of future studies.

More evidence for post-translational regulation of Git1p activity is provided by observing wild type transport activity as a function of time after transfer of cells to low P$_i$, medium. The increase and then decline in activity (Fig. 3A) are consistent with the results of the Western blot (Fig. 4) and suggest that Git1p may be regulated at the level of protein turnover or stability. Many yeast plasma membrane permeases undergo regulated endocytosis and vacuolar degradation (18, 25) and/or are regulated by phosphorylation (26). The role of regulated degradation in controlling Git1p activity is currently being investigated.

\textbf{FIG. 6. GroPIns transport activity requires acidic pH.} The GroPIns transport activity of the wild type strain grown in I$^-$, low P$_i$ conditions was assayed as described under "Experimental Procedures" using the indicated buffers at a concentration of 100 mM. Values represent mean $\pm$ S.E. of triplicate determinations.

\textbf{FIG. 7. GroPIns can act as sole phosphate source.} Strains grown in I$^-$, low P$_i$, medium were harvested and suspended to equivalent cell densities in sterile water. Three 10-fold serial dilutions were made for each cell suspension. Each cell suspension (5 $\mu$L) was spotted onto plates lacking (I$^-$) or containing (I$^+$) 75 $\mu$M inositol, containing no source of phosphate (no P$_i$), containing 75 $\mu$M KH$_2$PO$_4$, or containing 75 $\mu$M GroPIns.
The GIT1 promoter region (Fig. 5) contains potential Pho4p (CACGTG) and Pho2p (TAATRA/TAANTAA) binding sites (21). Removal of the DNA containing three potential Pho4p binding sites (~640 and ~330 relative to the start codon) abolishes transcription induction in response to phosphate limitation in both the presence and absence of inositol (Fig. 5). Pho4p, like Ino2p and Ino4p, are bHLH binding proteins. The GIT1 promoter does not contain a copy of the UASINO core consensus sequence (CACGTG) but does contain a bHLH consensus sequence (CACGGT) to which Ino2p and Ino4p have been shown to bind to minimally activate INO1 transcription (22). Some bHLH binding proteins are known to form multiple dimer combinations that act upon diverse sets of genes (23). Indeed, Ino4p has been shown by yeast two-hybrid and biochemical assays to interact with Pho4p (24). Thus, it is tempting to speculate that Pho4p may heterodimerize with Ino2p or Ino4p to activate GIT1 transcription. The actual mechanism of regulation, however, must be more complex, as neither Ino2p nor Ino4p is required for transcriptional induction in cells grown in inositol-containing medium, and all four transcription factors are required when cells are grown in low P_i medium. Clearly, further studies are required to elucidate the details of GIT1 transcriptional regulation and to prove a direct interaction between Pho4p, or any other DNA-binding protein, and the GIT1 promoter. We showed that inositol and phosphate do not effectively compete with GroPIns for transport by the Git1p permease. Neither a 200-fold excess of P_i nor a 200-fold excess of inositol inhibits GroPIns transport in short term transport assays (Table II) (12). Others have reported that a strain in which all known phosphate transporter genes (PHO84, PHO87, PHO89, PHO90, PHO91) have been deleted is viable in high P_i medium if GIT1 is overexpressed but not when GIT1 is present in single copy (27). Because the medium did not contain exogenous GroPIns (27), Git1p must have some limited affinity for P_i. Like the major phosphate transporter, Pho84p (2), Git1p appears to act as a H^+ symporter. The evidence for H^+ -coupled uptake is the observed decrease in transport activity with increasing buffer pH (Fig. 6) and the abolishment of transport activity by the protonophore carbonyl cyanide m-chlorophenylhydrazone (Table II).

In previous studies, we have shown that GroPIns can act as the sole source of inositol (12). Remarkably, we report that GroPIns can also act as the sole source of phosphate for the cell in a manner that supports robust growth (Fig. 7). In fact, there is no discernable difference in growth when the wild type strain is supplied with 75 nM GroPIns as compared with 75 nM KH2PO4. To our knowledge, this is the first demonstration of a phosphate-containing organic molecule (GroPIns) acting as the sole phosphate source for a eukaryotic cell. Indeed, the inositol auxotrophic strains, ino2Δ and ino4Δ, grow robustly when GroPIns is acting as the sole source of both inositol and phosphate (Fig. 7).

Through the action of phospholipase B, S. cerevisiae hydrolyzes external phosphatidylinositol to release GroPIns (13). Thus, the combination of external phospholipases and Git1p provide yeast with the capability of obtaining crucial nutrients (inositol and phosphate) from environments in which P_i or its deacylation product, GroPIns, are available. In a natural environment, the source of P_i or GroPIns may be such things as decaying fruit or plant exudates (28). Once inside the cell, GroPIns must be metabolized to liberate inositol and phosphate (Fig. 1). The gene products involved likely include one or more glycerophosphodiesterases and glycerol phosphatases. We are in the process of determining the genes and enzymatic activities responsible for this metabolism.

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