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Production and Reutilization of an Extracellular Phosphatidylinositol Catabolite, Glycerophosphoinositol, by *Saccharomyces cerevisiae*

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Phosphatidylinositol catabolism in *Saccharomyces cerevisiae* is known to result in the formation of extracellular glycerophosphoinositol (GroPIns). We now report that *S. cerevisiae* not only produces but also reutilizes extracellular GroPIns and that these processes are regulated in response to inositol availability. A wild-type strain uniformly prelabeled with [^{3}H]inositol displayed dramatically higher extracellular GroPIns levels when cultured in medium containing inositol than when cultured in medium lacking inositol. This difference in extracellular accumulation of GroPIns in response to inositol availability was shown to be a result of both regulated production and regulated reutilization. In a strain in which a negative regulator of phospholipid and inositol biosynthesis had been deleted (an *opi1* mutant), this pattern of extracellular GroPIns accumulation in response to inositol availability was altered. An inositol permease mutant (*itr1 itr2*), which is unable to transport free inositol, was able to incorporate label from exogenous glycerophospho[^{3}H]inositol, indicating that the inositol label did not enter the cell solely via the transporters encoded by *itr1* and *itr2*. Kinetic studies of a wild-type strain and an *itr1 itr2* mutant strain revealed that at least two mechanisms exist for the utilization of exogenous GroPIns: an inositol transporter-dependent mechanism and an inositol transporter-independent mechanism. The inositol transporter-independent pathway of exogenous GroPIns utilization displayed saturation kinetics and was energy dependent. Labeling studies employing [^{14}C]glycerophospho[^{3}H] inositol indicated that, while GroPIns enters the cell intact, the inositol moiety but not the glycerol moiety is incorporated into lipids.

The extracellular production of glycerophosphoinositol (GroPIns) represents a major pathway of phosphatidylinositol (PI) metabolism in growing cultures of *Saccharomyces cerevisiae* (1, 2). GroPIns accumulates in the growth medium at levels equivalent to about 25% of the amount of cellular PI, in contrast to much lower extracellular levels of the deacylated forms of the major yeast phospholipids phosphatidylcholine and phosphatidylethanolamine (1). In pulse-chase turnover experiments, Angus and Lester (1) have shown that GroPIns accounts for approximately 50% of the phosphorus and inositol lost from PI during growth. Furthermore, the release of GroPIns is regulated by glucose in the medium (2). Thus, the extracellular accumulation of GroPIns was shown to be specific, regulated, and a major route of PI turnover in a growing yeast culture. Recently, Hawkins et al. (9) confirmed that the addition of glucose to stationary-phase cultures results in the extracellular production of not only GroPIns but also low levels of GroPIns 4-phosphate and GroPIns 4,5-bisphosphate.

GroPIns, GroPIns 4-phosphate, and GroPIns 4,5-bisphosphate are produced from PI, PI 4-phosphate, and PI 4,5-bisphosphate, respectively, by a phospholipase A and a lysophospholipase acting sequentially or by a phospholipase B. Phospholipase B activities have been found in the plasma membrane, the periplasmic space, and in the medium of *S. cerevisiae* (11, 21–23). Recently, a gene (*PLB1*) encoding a protein predicted to have a 45% amino acid sequence identity with phospholipase B from *Penicillium notatum* has been isolated from *S. cerevisiae* (13). A *plb1* deletion mutant releases greatly reduced levels of the phosphatidylcholine and phosphatidylethanolamine deacylation products, glycerophosphocholine and glycerophosphoethanolamine, into the medium but releases wild-type levels of the PI catabolite, GroPIns. Thus, *PLB1* does not appear to encode the phospholipase primarily responsible for the extracellular production of GroPIns. The enzyme(s) responsible for this activity in *S. cerevisiae* has yet to be characterized.

Previous studies of the inositol-requiring yeast *Saccharomyces uvarum* revealed that released GroPIns is reutilized by inositol-starved but not inositol-supplemented cells (18). Exogenous GroPIns was shown to be hydrolyzed by inositol-starved *S. uvarum* cells into glycerol, P_i, and inositol, presumably by the combined action of a phosphodiesterase and a phosphomonoesterase. In addition, it was demonstrated that, during incubation with glycerol[^{32}P]phospho[^{3}H]inositol, both [^{32}P]phosphate and [^{3}H]inositol are taken up by inositol-starved cells (18).

S. cerevisiae both synthesizes and transports inositol (20). The rate-limiting step in inositol biosynthesis is catalyzed by inositol-1-phosphate synthase, which is encoded by the *INO1* gene (7). Two inositol transporter genes, *ITR1* and *ITR2*, have been identified (16, 17). Expression of both the *INO1* gene (10) and the major inositol permease, the *ITR1* gene (12), is downregulated in response to the availability of inositol. This pattern of regulation is similar to the regulation exhibited by enzymes involved in phospholipid biosynthesis in *S. cerevisiae*. The transcriptional regulation of the phospholipid biosynthetic genes, *INO1*, and *ITR1* occurs through the action of the products of the *INO2*, *INO4*, and *OPI1* genes, which were originally identified on the basis of their ability to regulate phospholipid biosynthesis (7). In this study, we have investigated the regulation of the extracellular accumulation and reutilization of GroPIns in response to inositol availability in a wild-type *S. cerevisiae* strain and in a mutant defective in the regulation of phospholipid biosynthesis (an *opi1* mutant) (19). In addition, we have utilized a mutant defective in inositol uptake (*itr1 itr2*

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mutant) in order to investigate the mechanism(s) of the utilization of extracellular GroPIns by *S. cerevisiae*.

MATERIALS AND METHODS

Materials. Materials and sources are as follows: *myo*-[2-^{3}H]inositol, *myo*-[2-^{3}H]inositol-phosphate, glycerophospho-*myo*-[2-^{3}H]inositol, and [U-^{14}C]glycerol, American Radiochemicals Inc.; Dowex-1 resin, GroPIns, and inositol-phosphate, Sigma; Chelex 100, Bio-Rex 70, and AG4 resins, Bio-Rad; Celite 545, Fisher; GF/A glass fiber filters and SG81 paper, Whatman; white ribbon paper 589, Schleicher & Schuell.

Strains and culture conditions. The following laboratory strains of *S. cerevisiae* were used: DC5 (*his3-11,15 leu2-3,-112 MAT***a**) and *opi1* (*opi1*::*LEU2 leu2-3,-112 his3-11,-15 MAT***a**). The strains FY250 (*trp1 leu2 his3 ura3 MAT***a**) and *itr1 itr2* (*itr1*::*TRP1 itr2*::*HIS3 trp1 leu2 his3 ura3*) were generously provided by P. McGraw (University of Maryland). DC5 was used as the wild-type control in experiments involving the *opi1* mutant. FY250 was used as the wild-type control in experiments involving the *itr1 itr2* mutant. Strains were grown aerobically at 30°C with shaking. Turbidity was monitored by measurement of A_{650} on a Beckman DU 64 spectrophotometer. In the experiments described, mid-log phase refers to approximately 3×10^6 to 3×10^7 cells per ml ($A_{650} \approx 0.1$ to 1) and stationary phase refers to approximately 10^8 cells per ml ($A_{650} \geq 2$). The synthetic complete media used consisted of (per liter): 30 g of glucose, 5 g of ammonium sulfate, 1 g of potassium phosphate (monobasic), 0.5 g of magnesium sulfate, 0.1 g of sodium chloride, 0.1 g of calcium chloride, 0.5 mg of boric acid, 0.04 mg of cupric sulfate, 0.1 mg of potassium iodide, 0.2 mg of ferric chloride, 0.4 mg of manganese sulfate, 0.2 mg of sodium molybdate, 0.4 mg of zinc sulfate, 20 mg of adenine, 20 mg of arginine, 20 mg of histidine, 60 mg of leucine, 230 mg of lysine, 20 mg of methionine, 300 mg of threonine, 20 mg of tryptophan, 40 mg of uracil, 2 μg of biotin, 400 μg of pantothenate, 2 μg of folic acid, 400 μg of niacin, 200 μg of *p*-aminobenzoic acid, 400 μg of pyridoxine hydrochloride. Some media were supplemented with the indicated amounts of *myo*-inositol or GroPIns.

Preparation of [^{14}C]glycerophospho[^{3}H]inositol. Strain AW303 was grown on complex medium consisting of 1% yeast extract, 1% Bacto Peptone, 0.5% KH_2PO_4, and 2% ethanol and supplemented with 5 μCi of [U-^{14}C]glycerol and 2.5 μCi of *myo*-[2-^{3}H]inositol per ml. The culture was harvested in stationary phase, and the cell pellet was suspended in 5% trichloroacetic acid and placed on ice for 15 min. After being washed twice with H_2O, the pellet was extracted with 1 ml ethanol-diethyl ether-H_2O-pyridine-concentrated NH_4OH (15:5:15:1:0.018, vol/vol) (solvent 1) for 1 h at 60°C (8). A lipid fraction free of nonlipids was prepared as described by Becker and Lester (4) except that the alkaline methanol was immediately neutralized to avoid deacylation of the glycerophospholipids. The lipid fraction was dried down and resuspended in 1 ml of solvent 1. The acidic lipids were separated from the neutral lipids as follows (15). The sample was added to a column consisting of 0.5 ml each of AG4 (X4, 100 to 200 mesh, free base form) and Chelex 100 (200 to 400 mesh, sodium form) resins equilibrated with methanol. The neutral lipids were eluted with 1 ml of methanol and 5 ml of chloroform-methanol (1:1). The acidic lipids were eluted with 4 ml chloroform-methanol-water (16:16:5, vol/vol) containing 0.4 N NH_4OH. After drying down and resuspension in chloroform-methanol-water (16:16:5, vol/vol), the sample was spotted onto a silica gel thin-layer chromatography plate and chromatographed in chloroform–methanol–4 N NH_4OH (9:7:2, vol/vol). The lipids were visualized by iodine vapor. The band running at the same R_f as standard PI (Sigma) was marked and scraped into a test tube following evaporation of the iodine under a hood. The PI was eluted from the silica gel with chloroform-methanol-water (16:16:5), the tube was centrifuged to sediment the silica gel, and the solvent containing PI was dried down under N_2. The sample was then resuspended in 0.5 ml of methanol-toluene (1:1) and subjected to mild alkaline methanolysis as described previously (14). The resulting GroPIns was desalted by adding it to a 0.7-ml column of Bio-Rex 70 (50 to 100 mesh, sodium form) and eluting with 1 ml of H_2O. The sample was lyophilized, resuspended in 0.2 ml of H_2O-methanol (1:1), and stored at −20°C.

Analysis of inositol-containing metabolites in the media. Cells were grown to uniform labeling in synthetic medium containing 75 μM inositol and 1 to 5 μCi of [^{3}H]inositol. While in mid-log phase, the cells were harvested, washed, and recultured to mid-log phase (approximately 3×10^6 cells per ml) in nonradioactive synthetic media either containing or lacking 75 μM inositol. In some cases, the cultures were supplemented with 100 μM nonradioactive GroPIns. At various times, the media were analyzed for GroPIns.

The separation of inositol-labeled metabolites was performed on 0.5 ml Dowex-1 (formate form, X8, 200 to 400 mesh) anion-exchange resins, as described previously (5). The supernatants of centrifuged culture media (0.5 ml) were diluted with 4.5 ml of H_2O and passed through the columns. The columns were washed with 2 to 3 ml of H_2O to remove all traces of free [^{3}H]inositol. [^{3}H]GroPIns was eluted from the resin with 3.0 ml of 5 mM sodium tetraborate–60 mM ammonium formate. Elution of more polar [^{3}H]inositol metabolites requires increasing concentrations of formic acid and ammonium formate: inositol phosphate, 0.1 M formic acid–0.2 M ammonium formate; inositol bisphosphate, 0.1 M formic acid–0.4 M ammonium formate; inositol trisphosphate, 0.1 M formic acid–1 M ammonium formate. This protocol was validated by using the appropriate ^{3}H-labeled standards. Liquid scintillation counting was carried out in a Beckman LS5801 liquid scintillation counter with Ecolume liquid scintillation cocktail (ICN Biomedicals).

Determination of label uptake from exogenous glycerophospho[2-^{3}H]inositol [2-^{3}H]inositol-phosphate, and [2-^{3}H]inositol. Label uptake into cells was determined by one of two methods. In the first method, 1-ml aliquots of cultures were centrifuged to sediment the cells. Cell pellets were washed twice with fresh medium, resuspended in H_2O, and subjected to liquid scintillation counting. Alternatively, 1-ml aliquots of the cultures were removed and filtered over glass fiber filters. The filters were washed with 15 ml of water and counted to quantify the amount of radioactivity incorporated into the cells.

Determination of label uptake from exogenous [^{14}C]glycerophospho[^{3}H]inositol. Wild-type and *itr1 itr2* cultures were inoculated at a low density (approximately 10^5 cells per ml) in 1.5 ml of medium supplemented with 75 μM [^{14}C]glycerophospho[^{3}H]inositol and lacking inositol. Following 30 h of growth (late log to early stationary phase), 0.5-ml samples of the cultures were centrifuged to sediment the cells, and the supernatants were saved. The cell pellets were washed twice in water, resuspended in 0.5 ml of water, and subjected to liquid scintillation counting.

Pulse-chase labeling with [^{14}C]glycerophospho[^{3}H]inositol. Cell cultures were grown to mid-log phase in media lacking inositol. The cells were harvested, washed with fresh media, and resuspended to an A_{650} of 5 (approximately 2×10^8 cells per ml) in fresh media. The cell suspension (800 μl) was incubated with 200 μl of 500 μM [^{14}C]glycerophospho[^{3}H]inositol for 20 min at 30°C with shaking. The cells were harvested, resuspended in 1 ml of fresh medium containing 40 μM unlabeled GroPIns, and incubated at 30°C with shaking. At time zero, 20 min, and 60 min after the initiation of the chase, 300-μl aliquots of the culture were centrifuged, the supernatants were saved, and the pellets were suspended in 3.5% perchloric acid. After 30 min on ice, the perchloric acid suspensions were centrifuged to pellet the cells. The supernatants were removed, neutralized with 3 M KOH, and saved as the perchloric acid extract. Both the acid extract and the media were chromatographed on Dowex-1 resin as described for the analysis of inositol-containing metabolites in the media. The identity of [^{14}C]glycerol was determined by its inability to bind to Dowex-1 resin and by paper chromatography on white ribbon paper (system III [2]). The pellet remaining following the acid extraction procedure was washed twice in water, suspended in 100 μl of ethanol-diethyl ether-H_2O-pyridine-concentrated NH_4OH (15:5:15:1:0.018, vol/vol), and incubated at 60°C for 30 min to extract lipids. To confirm that the extract radioactivity belonged to lipids, a portion of the sample was spotted onto SG81 paper and chromatographed in chloroform-methanol-4 N NH_4OH (9:7:2, vol/vol). In this system, water-soluble compounds remain at the origin and lipids migrate up the paper.

GroPIns transport assay. In order to measure initial rates of GroPIns transport for kinetic analysis, a short-term GroPIns transport assay was developed. The assay was developed with respect to uptake of radioactivity from glycerophospho[2-^{3}H]inositol into an *itr1 itr2* mutant (in order to examine GroPIns utilization occurring independently of the inositol permeases). In this strain, uptake versus time was linear for at least 20 min. Cell cultures were grown in the indicated media to mid-log phase (A_{650}, 0.6 to 1.0). Transport activity varied somewhat depending upon the growth stage at which the cells were harvested. The cells were harvested, washed twice with fresh media, and resuspended to an A_{650} of 5 (approximately 2×10^8 cells per ml) in fresh media. The cell suspension (200 μl) was incubated with 50 μl of glycerophospho[2-^{3}H]inositol and unlabeled GroPIns at the desired concentration. Assays were carried out for 15 min at 30°C with shaking. Following termination of assays by the addition of 10 ml of cold water, cells were collected by filtration through a glass fiber filter. The filters were washed with 15 ml of water and subjected to liquid scintillation counting. In some cases (see Table 3), changes were made to the assay conditions by the addition of 20 mM sodium azide or 400 μM 2,4-dinitrophenol in ethanol, by performing the assay at 10°C, or by the removal of glucose. For assays in which glucose was absent, the samples were preincubated at 30°C for 20 min before the addition of the substrate in order to partially deplete the cells of ATP.

RESULTS AND DISCUSSION

Effect of inositol availability on extracellular GroPIns levels. As a first step in defining the effect of inositol availability on the level of extracellular GroPIns, uniformly labeled wild-type and *opi1* cells were inoculated in media either lacking or containing inositol. The amount of GroPIns in the culture medium was monitored over time (Fig. 1A and C). Wild-type cells grown in the presence of inositol displayed increasing extracellular GroPIns levels for approximately three generations (mid-log phase) after inoculation in fresh medium, at which point the level of GroPIns reached a plateau. When wild-type cells were inoculated into fresh medium lacking inositol, there was a slight increase in extracellular GroPIns levels followed by a decrease to very low levels. In an *opi1* strain in which a

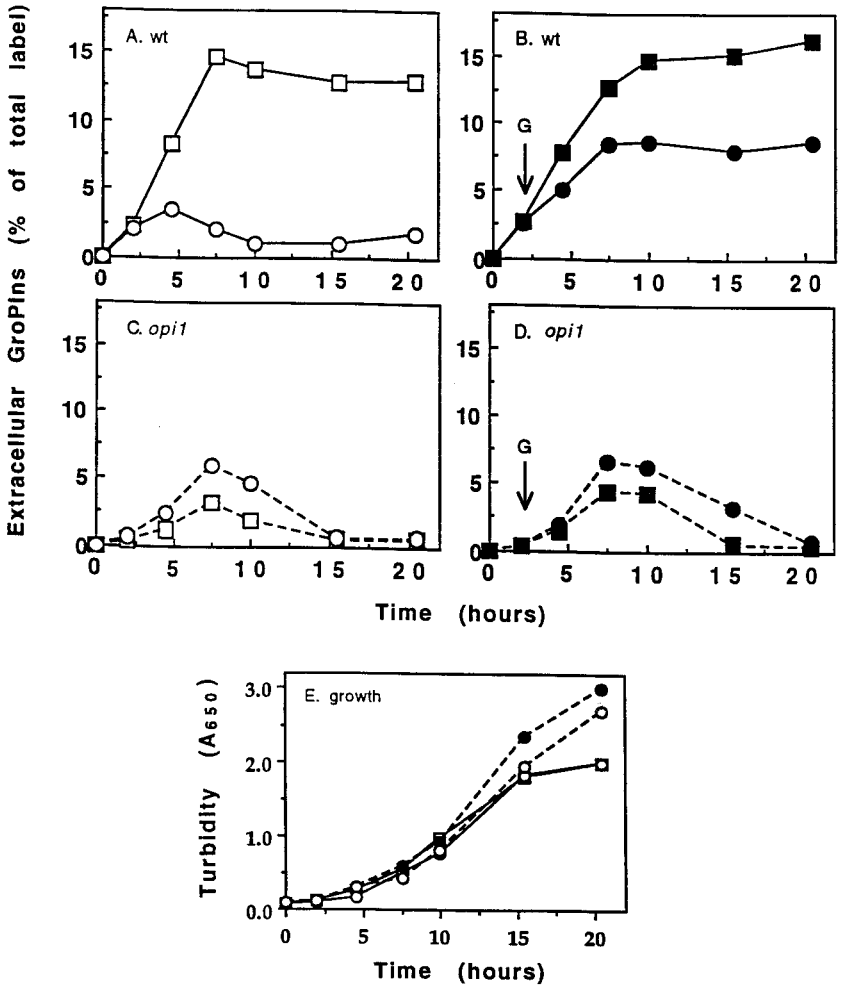

FIG. 1. Extracellular accumulation of GroPIns. Wild type (wt) and *opi1* strains were grown to uniform labeling with [2-^{3}H]inositol. At time zero, the cells were harvested, washed, and recultured in nonradioactive synthetic medium either containing or lacking 75 μm inositol (A and C). Panels B and D are identical to panels A and C except that exogenous GroPIns (G) (100 μm) was added after 2 h of growth. At the indicated times, aliquots of the media were analyzed for GroPIns. Growth is shown in panel E. The experiment was repeated with similar results. □, cultures containing inositol; ○, cultures lacking inositol; ■, cultures containing inositol that were supplemented with exogenous GroPIns; ●, cultures lacking inositol that were supplemented with exogenous GroPIns. ——, wild-type strain; – – –, *opi1* strain.

negative regulator of phospholipid biosynthesis, inositol biosynthesis, and inositol transport had been deleted, the GroPIns levels similarly increased and then decreased (Fig. 1C). However, the same pattern occurred whether inositol was in the medium or not. The amount of radiolabeled GroPIns appearing in the medium of the *opi1* culture, as a percentage of the total label incorporated into the cells, is difficult to compare with that for the wild type. The *opi1* strain exhibits higher transport activity than the wild type (12) and constitutively synthesizes inositol (7) and, therefore, is likely to have a specific activity of radiolabeled inositol that's different from that for the wild type. However, there is clearly an altered pattern of extracellular GroPIns accumulation in the *opi1* strain in response to inositol availability.

The decrease in GroPIns levels in Fig. 1A and C at later time points suggested that there is a mechanism for the breakdown and/or reutilization of GroPIns. Thus, the overall extracellular accumulation of GroPIns is the result of two consequent processes, PI deacylation and GroPIns reutilization.

Effect of inositol availability on GroPIns production. In order to separate the production from the reutilization of extracellular GroPIns, experiments in which the media were supplemented with 100 μM unlabeled GroPIns were performed. The conditions used for this experiment were identical to those of Fig. 1A and C except that exogenous, unlabeled GroPIns was added after 2 h of growth (Fig. 1B and D). Clearly, the presence of unlabeled GroPIns resulted in increased accumulation of the labeled GroPIns in the media of wild-type cells lacking inositol (Fig. 1B). Presumably, this occurs because the unlabeled GroPIns competes with the radiolabeled GroPIns for uptake. In the presence of inositol, the addition of unlabeled GroPIns to wild-type cultures had little effect. The addition of unlabeled GroPIns to the *opi1* cultures resulted in only a slight increase in extracellular radiolabeled GroPIns levels.

Cultures grown in the presence of inositol have been shown to produce approximately 1 μM extracellular GroPIns per A_{650} of ≈1 (1). Therefore, after 10 h of growth under these conditions the amount of GroPIns produced by the cells should have been negligible (≈1 μm) compared with the 100 μM exogenously added GroPIns. By using data from an uptake experiment to be discussed later (see Table 1), it can be calculated that, at most, 6 μM GroPIns per A_{650} of ≈1 is utilized by wild-type cells in the absence of inositol. Assuming that the

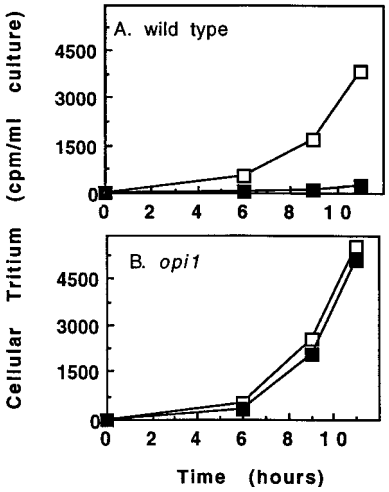

FIG. 2. Uptake of label from exogenous glycerophospho[2-^{3}H]inositol into wild-type and *opi1* strains. Strains were inoculated at a low density (10^5 cells per ml) in media either containing (■) or lacking (□) 75 μM inositol and supplemented with exogenous carrier-free glycerophospho[2-^{3}H]inositol (5 Ci/mmol). At the indicated times, aliquots of the cultures were centrifuged. Cell pellets were washed and subjected to liquid scintillation counting. The experiment was repeated with similar results.

labeled GroPIns, which is produced by the cells, mixes with the exogenous unlabeled GroPIns, at most 5 μM or 5% of the label should have reentered the cell through utilization between 2 and 10 h of growth (a change in A_{650} of ≈0.8). Thus, the amount of radioactive GroPIns in the medium of the wild-type strain between 2 and 10 (Fig. 1B and D) should approximate the amount of GroPIns produced by the cells during that span of logarithmic growth and should not reflect a combination of production and reutilization. The data suggest that wild-type strains produce more GroPIns when inositol is present in the media than when inositol is absent. The production of GroPIns in the *opi1* strain (Fig. 1D), however, did not display this regulation.

Effect of inositol on GroPIns utilization. In order to analyze the possible reutilization of the GroPIns molecule, experiments in which the uptake of label from exogenously added glycerophospho[^{3}H]inositol was monitored over time were performed (Fig. 2). Carrier-free glycerophospho[^{3}H]inositol was used in this experiment (5 Ci/mM), although the qualitative results were identical with 75 μM GroPIns (data not shown). When inositol was present in the media, wild-type cells incorporated little or no label from glycerophospho[^{3}H]inositol. However, when inositol was absent, wild-type cells became labeled (Fig. 2A). Both in the presence and in the absence of inositol, *opi1* mutants incorporated the label from exogenously added glycerophospho[^{3}H]inositol (Fig. 2B).

In order to interpret these data, it was necessary to define the mechanism of GroPIns utilization. For example, if GroPIns could be transported by the inositol transporters or if yeast cells were utilizing GroPIns by hydrolyzing it extracellularly to free inositol before uptake, the inositol in the medium could simply compete with the radiolabeled molecules for uptake, thus slowing apparent reutilization. However, the fact that the *opi1* mutant incorporated nearly identical amounts of label in both the presence and the absence of inositol argues against a simple dilution effect.

Uptake of label from [^{3}H]inositol, [^{3}H]inositol-phosphate, and glycerophospho[^{3}H]inositol. The previous experiments indicated that, at a minimum, the inositol moiety of GroPIns was

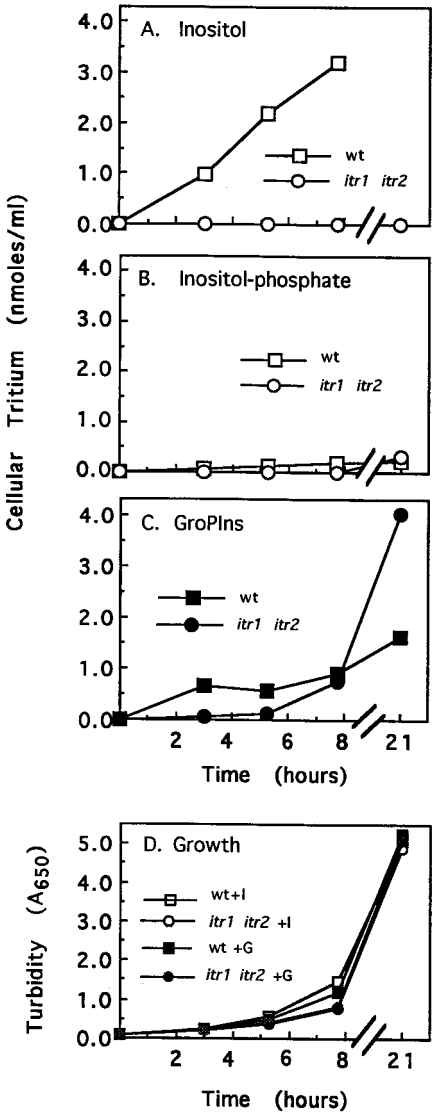

FIG. 3. Uptake of label from exogenous [2-^{3}H]inositol (A), [2-^{3}H]inositol-phosphate (B), or glycerophospho[2-^{3}H]inositol (GroPIns) (C) into wild-type (wt) and *itr1 itr2* strains. Strains were grown to mid-log phase in synthetic media lacking inositol. The cells were harvested, washed, and recultured to mid-log phase in synthetic media containing either 10 μM glycerophospho[2-^{3}H]inositol (G), 10 μM [2-^{3}H]inositol-phosphate, or 10 μM [2-^{3}H]inositol (I). At indicated times, aliquots of the cultures were filtered and subjected to liquid scintillation counting. (D) Growth data for experiments of panels A and C. Note the interruption of the *x*-axis time scale.

being utilized by the cell. To study the substrate specificity of this label uptake, the incorporation of exogenous [^{3}H]inositol, [^{3}H]inositol-phosphate, and glycerophospho[^{3}H]inositol was analyzed over time in a wild-type strain and in a strain carrying disruptions in both of the known inositol permease genes (an *itr1 itr2* mutant [12]) (Fig. 3). The inositol permease mutant was incapable of incorporating label from free [^{3}H]inositol (Fig. 3A), as previously shown (12). However, both strains were able to incorporate radioactivity from glycerophospho [^{3}H]inositol (Fig. 3C). Although the rate of label uptake appeared to be slower in the inositol permease mutant, over the course of the experiment (20 h) the inositol permease mutant actually took up more label from glycerophospho[^{3}H]inositol

TABLE 1. Incorporation of [^{3}H]inositol and [^{14}C]glycerol from exogenous [^{14}C]glycerophospho[^{3}H]inositol into a wild-type strain and an inositol permease mutant[a]

Strain	OD_{650}[b]	Concn in cell pellet (nmol/ml)[c]	
		Inositol	Glycerol
Wild type	3.0	6.0	0.0
	1.7	8.5	0.3
itr1 itr2	4.0	20.3	1.7
	3.4	19.3	2.4

[a] Wild-type and *itr1 itr2* strains were inoculated into inositol-free media supplemented with 75 μM [^{14}C]glycerophospho[^{3}H]inositol. After 30 h of growth (late log to early stationary phase), cultures were centrifuged. The cell pellets were washed, resuspended in water, and subjected to liquid scintillation counting.

[b] OD_{650}, optical density at 650 nm.

[c] From two experiments in which cells were harvested at different densities. The data were calculated from known specific activities.

TABLE 2. Incorporation of [^{3}H]inositol and [^{14}C]glycerol from exogenous [^{14}C]glycerophospho[^{3}H]inositol into a wild-type strain and an inositol permease mutant[a]

Strain and chase (min)	% of total cpm[b]					
	Medium		$HClO_4$ extract		Lipid	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Wild type						
0	0.0	0.0	58.0	93.6	42.0	6.4
20	2.0	21.0	32.8	71.1	65.2	7.9
60	2.8	36.9	16.0	50.0	81.2	13.1
itr1 itr2						
0	0.0	0.0	81.0	97.0	19.0	3.0
20	6.4	31.9	44.0	62.5	49.6	5.6
60	7.8	39.7	22.7	53.6	69.5	6.6

[a] Wild-type and *itr1 itr2* strains pulsed for 20 min with [^{14}C]glycerophospho[^{3}H]inositol were washed and suspended in fresh media containing 40 μM unlabeled GroPIns. At the indicated times, aliquots of the assay mixtures were removed and both the ^{3}H and the ^{14}C cpm in the medium, the $HClO_4$ extract, and the lipid fraction were determined.

[b] After a 20-min pulse. The data are representative of two independent experiments.

than the wild-type strain. Little uptake of [^{3}H]inositol-phosphate occurred in either strain (Fig. 3B).

These results indicate that there exists a mechanism for utilizing GroPIns independent of the inositol permeases. Furthermore, this mechanism appears to be specific for GroPIns compared with inositol and inositol-phosphate.

Long-term uptake of label from [^{14}C]glycerophospho[^{3}H]inositol. A long-term uptake experiment was performed in order to determine the final fate of the inositol and glycerol moieties derived from [^{14}C]glycerophospho[^{3}H]inositol (Table 1). Wild-type and *itr1 itr2* strains incorporated [^{3}H]inositol label but little or no [^{14}C]glycerol label into the cell from exogenously added [^{14}C]glycerophospho[^{3}H]inositol; at least 10-fold more inositol than glycerol was incorporated into the cells. These experiments were performed with 75 μM GroPIns in order to allow for a calculation of the amount of GroPIns being utilized by the cell under the indicated conditions. In the absence of unlabeled carrier GroPIns, the qualitative results were identical (data not shown); there was little or no net accumulation of the glycerol moiety into the cell, and the inositol label entered the cell in both the wild type and the *itr1 itr2* strains. These results suggested at least two possibilities. (i) GroPIns is hydrolyzed outside the cell. In this scenario, the inositol moiety enters the cell, but the glycerol moiety does not. (ii) GroPIns is hydrolyzed during or after transport. In this case, the inositol moiety is incorporated into the cell, while the glycerol moiety either is not taken up or diffuses back out during the course of the experiment. With regard to the inositol permease mutant, only the second scenario is possible because free inositol clearly does not enter the cell (Fig. 3A). Whatever the mechanism of uptake, these data indicate that the cell hydrolyzes GroPIns under the conditions of this experiment. These data, together with those of Fig. 3, clearly indicate that the inositol label derived from GroPIns can be imported via a mechanism independent of the inositol permeases.

Pulse-chase labeling with [^{14}C]glycerophospho[^{3}H]inositol. A short-term pulse-chase assay allowed for a more detailed analysis of the mechanism by which GroPIns was being utilized by the cell (Table 2). Both wild-type and *itr1 itr2* cells were pulsed for 20 min with [^{14}C]glycerophospho[^{3}H]inositol, washed, and chased with nonradioactive GroPIns. At various times, the levels of radioactivity in the media, the acid-soluble fraction, and the lipid fraction were determined. In both strains, the chase with unlabeled GroPIns resulted in the movement of [^{3}H]inositol counts from the acid extract to the lipid fraction and the movement of [^{14}C]glycerol counts from the acid extract to the medium. The ^{14}C counts in the media appeared to be in the form of free [^{14}C]glycerol. The majority of radioactivity in the $HClO_4$ extract was in the form of [^{14}C]glycerophospho[^{3}H]inositol and [^{14}C]glycerol, with the amount of [^{14}C]glycerophospho[^{3}H]inositol decreasing as the chase progressed. The appearance of intact [^{14}C]glycerophospho[^{3}H]inositol in the acid-soluble fraction of the cell indicates that the GroPIns molecule enters the cell intact. Furthermore, the appearance of [^{14}C]glycerol in both the acid extract and the medium indicates that GroPIns is hydrolyzed intracellularly and that the free glycerol produced leaves the cell.

Label uptake from glycerophospho[^{3}H]inositol is derepressed by growth in the absence of inositol and inhibited by free inositol in a wild-type strain. In order to study the effect of growth in the presence or absence of inositol on transport velocity as well as the ability of inositol to compete with GroPIns for uptake, a short-term transport assay was developed. Tritium uptake from glycerophospho[^{3}H]inositol with an increasing GroPIns concentration was studied in the wild-type strain. Figure 4A displays the transport activity of cells pregrown without inositol and assayed either in the presence or in the absence of 1 mM inositol. The data show that the presence of inositol in the assay drastically decreased transport velocity. For the experiment in Fig. 4B, cells were pregrown in the presence of inositol and the effect of free inositol in the assay mixture was determined. Similarly, the presence of inositol in the assay mixture drastically decreased transport velocity. These two experiments suggest that free inositol competes with GroPIns for transport, presumably via the inositol transporters. Furthermore, a comparison of Fig. 4A and B demonstrates that cells grown in the absence of inositol display much greater transport activity than those grown in the presence of inositol (note the difference in *y*-axis scales), suggesting that GroPIns uptake is derepressed in the absence of inositol. This result is not unexpected given that the activity of the major inositol permease, ITR1p, is derepressed in the absence of inositol (12) and that some GroPIns uptake appears to utilize the inositol transporters.

Label uptake from glycerophospho[^{3}H]inositol is saturable in an *itr1 itr2* mutant. Figure 4C illustrates that saturable label

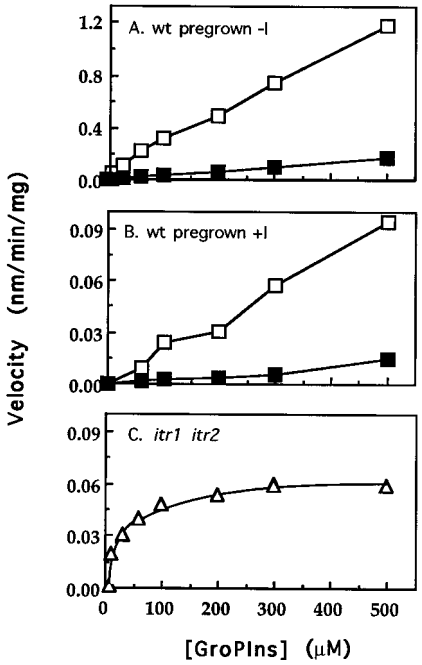

FIG. 4. Velocity of label uptake from exogenous glycerophospho[2-^{3}H]inositol (GroPIns) versus increasing glycerophospho[2-^{3}H]inositol concentration. The wild-type (wt) strain was pregrown either in the absence (A) or in the presence (B) of inositol, and the assay mixtures either contained (■) or lacked (□) 1 mM inositol. The *itr1 itr2* strain (C) was pregrown in the absence of inositol and assayed in the absence of inositol. Assays were carried out as described under "GroPIns transport assay." The concentration of GroPIns was varied as indicated. The data are means of duplicate determinations.

uptake occurs in an *itr1 itr2* mutant. The apparent K_m under these assay conditions was approximately 20 μM, and the V_{max} was approximately 0.06 nm/min/mg (dry weight) of cells. Pregrowth in the presence of inositol and the inclusion of inositol in the assay mixture had no effect upon transport velocity in the *itr1 itr2* mutant. Under the same assay conditions, the *itr1 itr2* mutant transported no [^{3}H]inositol, and neither the *itr1 itr2* mutant nor the wild-type strain incorporated appreciable label from [^{3}H]inositol-phosphate (data not shown). A comparison of Fig. 4A and C demonstrates that the wild-type strain was capable of much greater transport velocity than was the *itr1 itr2* mutant, again indicating that both an inositol transporter-dependent mechanism for GroPIns utilization and an independent one exist.

Label uptake from glycerophospho[^{3}H]inositol is temperature and energy dependent. The temperature dependence and energy dependence of label incorporation were studied (Table 3). Compared with the standard assay conditions (30°C), performing the assay at 10°C caused a substantial decrease in the velocity of label incorporation for both wild-type and *itr1 itr2* strains. Two presumptive proton conductors (3) were also tested (sodium azide and 2,4-dinitrophenol); both caused a substantial reduction in velocity of label incorporation. Likewise, performing the assay in the absence of glucose caused a decrease in label incorporation for both strains. These results are not unexpected for the wild-type strain given that GroPIns transport appears to utilize the inositol transporters (Fig. 3), which are known to be energy dependent. The *itr1 itr2* mutant, however, also exhibited an energy-dependent GroPIns transport capability. The effect of the energy inhibitors on GroPIns transport in the *itr1 itr2* strain appeared to be direct since 80 to 90% of the label could be found as intracellular GroPIns at the end of the 15-min assay.

TABLE 3. Energy and temperature dependence of label uptake from glycerophospho[2-^{3}H]inositol

Assay conditions	Velocity (pm/min/mg)	
	Wild type	*itr1 itr2*
Standard[a]	102	14
10°C	57	2
Sodium azide[b]	2	1
Ethanol[c]	89	8
DNP[d] in ethanol[c]	3	1
Preincubation[e]	117	7
Preincubation,[e] no glucose	67	2

[a] As described under "GroPIns transport assay."
[b] 20 mM.
[c] 5 μl.
[d] DNP, 2,4-dinitrophenol. Final concentration, 400 μM.
[e] 20 min at 30°C prior to the addition of the substrate.

Potential mechanism for GroPIns utilization. The uptake of glycerophosphodiesters in *Escherichia coli* provides a potential model for understanding the uptake of glycerophosphodiesters in *S. cerevisiae*. In *E. coli* glycerophosphodiesters are substrates of the *ugp*-encoded transport system which was originally recognized as a glycerol-3-phosphate (G3P) transport system (6). In this system, glycerophosphoryl diesters are thought to be hydrolyzed to glycerol-3-phosphate plus alcohol by a glycerophosphoryldiester phosphodiesterase which is closely associated with the transport system. In *E. coli*, this mechanism of hydrolysis associated with transport was hypothesized on the basis of experiments performed with glycerophosphoryl ethanolamine and glycerophosphorylcholine radiolabeled at various positions. For example, with glycerophosphoryl[^{3}H]choline as the substrate under conditions in which free choline could not be transported, label was taken up, but chemically unaltered glycerophosphoryl[^{3}H]choline could not be detected intracellularly. In addition, the hydrolytic products of glycerophosphoryl glycerol and glycerophosphoryl ethanolamine, glycerol and ethanolamine, were not detected intracellularly and were hypothesized by the authors to quickly diffuse out of the *E. coli* cell. In *S. cerevisiae*, we were able to detect GroPIns intracellularly, if transiently (Table 2), suggesting that hydrolysis may not be as tightly coupled to transport as it is in *E. coli*. Similarly to *E. coli*, however, the lack of net glycerol uptake from [^{14}C]glycerophospho[^{3}H]inositol over a long period (Table 1) is likely due to the free diffusion of glycerol across the membrane.

Concluding remarks. In this article, we report that the extracellular accumulation of GroPIns is regulated via the availability of inositol. Furthermore, the *OPI1* gene product plays a role in this regulation, suggesting that the extracellular accumulation of GroPIns is controlled in coordination with phospholipid and inositol biosynthesis and inositol transport in *S. cerevisiae*. Secondly, by utilizing an inositol permease mutant, we have found that at least two mechanisms exist for the utilization of exogenous GroPIns in *S. cerevisiae*: one mechanism is dependent upon the inositol transporters, and the second mechanism is independent of the inositol transporters. Finally, data obtained with [^{14}C]glycerophospho[^{3}H]inositol indicate that, while the entire GroPIns molecule enters the cell, the inositol moiety but not the glycerol moiety is incorporated into lipids.

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REFERENCES

1. **Angus, W. W., and R. L. Lester.** 1972. Turnover of inositol and phosphorus containing lipids in *Saccharomyces cerevisiae*; extracellular accumulation of glycerophosphoinositol derived from phosphatidylinositol. Arch. Biochem. Biophys. **151:**483–495.
2. **Angus, W. W., and R. L. Lester.** 1975. The regulated catabolism of endogenous and exogenous phosphatidylinositol by *Saccharomyces cerevisiae* leading to extracellular glycerophosphorylinositol and inositol. J. Biol. Chem. **250:**22–30.
3. **Ballarin-Denti, A., J. A. Den Hollander, D. Sanders, C. W. Slayman, and C. L. Slayman.** 1984. Kinetics and pH dependence of glycine-proton symport in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **778:**1–16.
4. **Becker, G. W., and R. L. Lester.** 1980. Biosynthesis of phosphoinositol-containing sphingolipids from phosphatidylinositol by a membrane preparation from *Saccharomyces cerevisiae*. J. Bacteriol. **142:**747–754.
5. **Berridge, M. J., R. C. M. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine.** 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. **212:**473–482.
6. **Brzoska, P., and W. Boos.** 1988. Characteristics of a *ugp*-encoded and *phoB*-dependent glycerophosphoryl diester phosphodiesterase which is physically dependent on the Ugp transport system of *Escherichia coli*. J. Bacteriol. **170:**4125–4135.
7. **Carman, G. M., and S. A. Henry.** 1989. Phospholipid biosynthesis in yeast. Annu. Rev. Biochem. **58:**635–669.
8. **Hanson, B. A., and R. L. Lester.** 1980. The extraction of inositol-containing phospholipids and phosphatidylcholine from *Saccharomyces cerevisiae* and *Neurospora crassa*. J. Lipid Res. **21:**309–315.
9. **Hawkins, P. T., L. R. Stephens, and J. R. Piggott.** 1993. Analysis of inositol metabolites produced by *Saccharomyces cerevisiae* in response to glucose stimulation. J. Biol. Chem. **268:**3374–3383.
10. **Hirsch, J. P., and S. A. Henry.** 1986. Expression of the *Saccharomyces cerevisiae* inositol-1-phosphate synthase (*INO1*) gene is regulated by factors that affect phospholipid synthesis. Mol. Cell. Biol. **6:**3320–3328.
11. **Ichimasa, M., T. Morooka, and T. Niimura.** 1984. Purification and properties of a membrane-bound phospholipase B from baker's yeast (*Saccharomyces cerevisiae*). J. Biochem. **95:**137–145.
12. **Lai, K., and P. McGraw.** 1994. Dual control of inositol transport in *Saccharomyces cerevisiae* by irreversible inactivation of permease and regulation of permease synthesis by *INO2*, *INO4*, and *OPI1*. J. Biol. Chem. **269:**2245–2251.
13. **Lee, K. S., J. L. Patton, M. Fido, L. K. Hines, S. D. Kohlwein, F. Paltauf, S. A. Henry, and D. E. Levin.** 1994. The *Saccharomyces cerevisiae PLB1* gene encodes a protein required for lysophospholipase and phospholipase B activity. J. Biol. Chem. **269:**19725–19730.
14. **Lester, R. L., and M. R. Steiner.** 1968. The occurrence of diphosphoinositide and triphosphoinositide in *Saccharomyces cerevisiae*. J. Biol. Chem. **243:**4889–4893.
15. **Lester, R. L., G. B. Wells, G. Oxford, and R. C. Dickson.** 1993. Mutant strains of *Saccharomyces cerevisiae* lacking sphingolipids synthesize novel inositol glycerophospholipids that mimic sphingolipid structures. J. Biol. Chem. **268:**845–856.
16. **Nikawa, J.-I., T. Nagumo, and S. Yamashita.** 1982. *myo*-Inositol transport in *Saccharomyces cerevisiae*. J. Bacteriol. **150:**441–446.
17. **Nikawa, J., Y. Tsukagoshi, and S. Yamashita.** 1991. Isolation and characterization of two distinct *myo*-inositol transporter genes of *Saccharomyces cerevisiae*. J. Biol. Chem. **266:**11184–11191.
18. **Paltauf, F., E. Zinser, and G. Daum.** 1985. Utilization of exogenous glycerophosphodiesters and glycerol-3-phosphate by inositol-starved yeast, *Saccharomyces uvarum*. Biochim. Biophys. Acta **835:**322–330.
19. **White, M. J., J. P. Hirsch, and S. A. Henry.** 1991. The OPI1 gene of *Saccharomyces cerevisiae*, a negative regulator of phospholipid biosynthesis, encodes a protein containing polyglutamine tracts and a leucine zipper. J. Biol. Chem. **266:**863–872.
20. **White, M. J., J. M. Lopes, and S. A. Henry.** 1991. Inositol metabolism in yeast. Adv. Microb. Physiol. **32:**1.
21. **Witt, W., H.-J. Bruller, G. Falker, and G. F. Fuhrmann.** 1982. Purification and properties of a phospholipid acyl hydrolase from plasma membranes of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **711:**403–410.
22. **Witt, W., A. Mertsching, and E. Konig.** 1984. Secretion of phospholipase B from *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **795:**117–124.
23. **Witt, W., M. E. Schweingruber, and A. Mertsching.** 1984. Phospholipase B from the plasma membrane of Saccharomyces cerevisiae. Separation of two forms with different carbohydrate content. Biochim. Biophys. Acta **795:**108–116.