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Coordinate Regulation of Phospholipid Biosynthesis in *Saccharomyces cerevisiae*: Pleiotropically Constitutive *opi1* Mutant

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Phospholipid metabolism in the *Saccharomyces cerevisiae opi1* mutant, which excretes inositol and is constitutive for the biosynthetic enzyme inositol-1-phosphate synthase (M. Greenberg, P. Goldwasser, and S. Henry, Mol. Gen. Genet. 186:157–163, 1982), was examined and compared to that of a wild-type strain. In wild-type *S. cerevisiae*, the phospholipid composition and the relative rates of synthesis of individual phospholipids change in response to the availability of exogenous supplies of soluble phospholipid precursors, particularly inositol. The *opi1* mutant, in contrast, displays a relatively invariant phospholipid composition, and its pattern of phospholipid synthesis does not change in response to exogenous phospholipid precursors. Phosphatidylinositol synthase was not found to be regulated in either wild-type or *opi1* cells. In wild-type cells, phosphatidylserine synthase and the phospholipid *N*-methyltransferases are coordinately repressed in response to a combination of inositol and choline. However, in *opi1* cells these activities are expressed constitutively. These results suggest that the gene product of the *OPI1* locus participates in the coordinate regulation of phospholipid synthesis.

In *Saccharomyces cerevisiae*, the synthesis of membrane phospholipids is regulated in a coordinate fashion (18). A number of enzymatic activities involved in the synthesis of phospholipids or their precursors (Fig. 1) are regulated in response to the soluble precursors, inositol and choline. The membrane-associated phospholipid *N*-methyltransferases which convert phosphatidylethanolamine (PE) to phosphatidylcholine (PC) (25, 26), phosphatidylserine (PS) synthase (6), and PS decarboxylase (7) are all repressed by the addition of exogenous choline to media already containing inositol. The presence of inositol in the growth medium is necessary for the repression of the phospholipid *N*-methyltransferases by choline (17, 27). When inositol is absent, choline has no effect upon *N*-methyltransferase activity.

Additional evidence of coordinate regulation of phospholipid biosynthesis in yeasts has come from the analysis of mutants. Many of the mutants with defects in the regulation of phospholipid metabolism were originally isolated on the basis of a defect in the regulation of the cytoplasmic enzyme inositol-1-phosphate synthase. This enzyme is responsible for the conversion of glucose-6-phosphate to inositol-1-phosphate, the precursor of inositol, which is in turn the precursor of phosphatidylinositol (PI) (Fig. 1). The enzyme inositol-1-phosphate synthase, which is the gene product of the *INO1* locus (11, 20), is repressed by exogenous inositol (8, 11). The *ino2* and *ino4* mutants, originally isolated as inositol auxotrophs (10, 12), are unable to derepress inositol-1-phosphate synthase despite the presence of a nonmutated copy of its structural gene, *INO1* (9, 11, 20). Thus, the *INO2* and *INO4* genes were identified as regulatory genes whose wild-type gene product is required for the expression of the *INO1* gene product (11, 23). Recently, however, the *ino2* and *ino4* mutants have been shown to have pleiotropic phenotypes. Also, in addition to their failure to express inositol-1-phosphate synthase, the *ino2* and *ino4* mutants are unable to derepress the membrane-associated phospholipid *N*-methyltransferases (23) which convert PE to PC (Fig. 1). These results suggest that inositol-1-phosphate synthase and the *N*-methyltransferases are coordinately regulated and that the wild-type gene products of the *INO2* and *INO4* loci participate as positive regulators in the coordinate regulation.

The finding that the *INO2* and *INO4* loci are involved in coordinate regulation of phospholipid synthesis has prompted us to reexamine other regulatory mutants which were originally isolated on the basis of altered regulation of inositol-1-phosphate synthase. The *opi* (*o*verproduction of *i*nositol) mutants were isolated on the basis of an inositol excretion phenotype (15) and were shown to be constitutive for inositol-1-phosphate synthase (14). In this study we examined the regulation of other enzymatic activities involved in phospholipid synthesis which are coordinately regulated by inositol and choline, including the phospholipid *N*-methyltransferase and phosphatidylserine synthase activities. These activities are shown to be constitutive in the *opi1* mutant, suggesting that the gene product of the *OPI1* locus participates in the coordinate regulation of phospholipid synthesis.

MATERIALS AND METHODS

Yeast strains and growth conditions. The isolation and inositol excretion phenotype of the *opi1-1* mutant has been previously described (14, 15). The wild-type strain (*ade5* **a**) is Opi$^+$ and does not excrete inositol (14, 15). In this study the mutant allele *opi1-1* was used for all experiments. A second allele, *opi1-12*, has been described (15), but its phenotype with respect to inositol-1-phosphate synthase constitutivity is somewhat leaky. It was therefore not used in this study.

Strains were maintained on YEPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Synthetic complete medium lacking inositol (10) contained the following: 2% glucose; 0.67% vitamin-free yeast base (Difco Laboratories); amino acids (lysine [20 mg/liter], arginine [10 mg/liter], leucine [10 mg/liter], methionine [10 mg/liter], adenine [10 mg/liter], uracil [10 mg/liter]); and vitamins (biotin [2 μg/liter],

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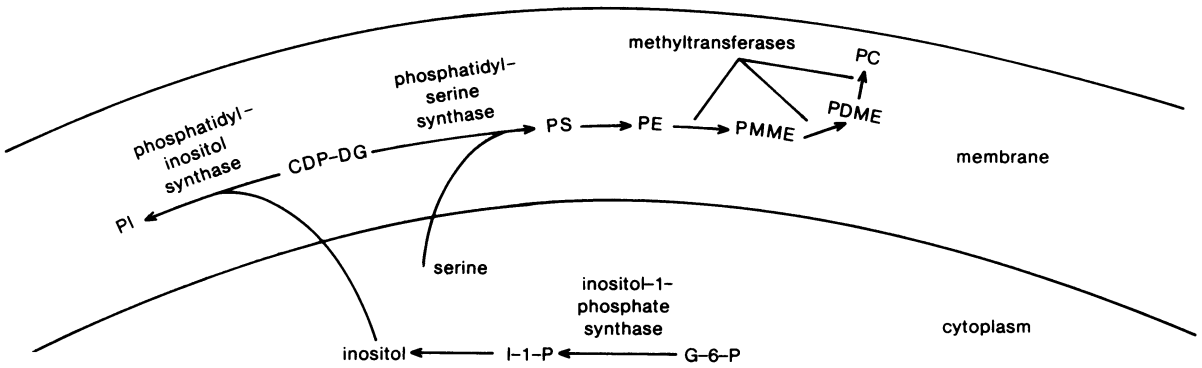

FIG. 1. Phospholipid synthesis in yeast cells. The reactions depicted in this diagram are discussed in the text. The location of each reaction in the membrane or the cytoplasm is illustrated by its position in the diagram. In yeast cells, PI and PS are synthesized in the membrane from a common precursor, cytidine diphosphate-diacylglycerol (CDP-DG). PS is decarboxylated to form PE, which undergoes three sequential methylations to form PC. The two intermediates in the formation of PC from PE are PMME and PDME. These reactions in *S. cerevisiae* were described by Steiner and Lester (24) and Waechter and Lester (25). The phospholipid precursor inositol is synthesized in the cytoplasm. Glucose-6-phosphate (G-6-P) is converted to inositol-1-phosphate synthase (I-1-P), which is subsequently dephosphorylated to form free inositol. This reaction series in *S. cerevisiae* was described by Culbertson et al. (8). Many other reactions involved in phospholipid synthesis in yeast cells, including the formation of PC from exogenous choline as described by Kennedy and Weiss (19), are not illustrated here, primarily because they are not the subject of this study.

calcium pantothenate [400 μg/liter], folic acid [2 μg/liter], niacin [400 μg/liter], *p*-aminobenzoic acid [200 μg/liter], pyridoxine hydrochloride [400 μg/liter]). Inositol (50 μM) and choline (1 mM) were added as indicated.

Cells used in immunoprecipitation analysis, labeling, or enzyme activity studies were pregrown overnight to a density of 5×10^6 to 1×10^7 cells per ml at 30°C in medium containing the various supplements. Optical density was determined by using a Klett spectrophotometer with a red filter. The number of cells per milliliter was determined by counting under a microscope on a hemacytometer.

Lipid labeling, extraction, and separation. For lipid composition analysis, cells were labeled with $^{32}P_i$ (New England Nuclear Corp.) in synthetic complete medium supplemented as described above. The final specific activity of the $^{32}P_i$ in the growth medium was 0.544 mCi/mmol. The cells were grown at 30°C for five to six generations in the presence of the label and then harvested in logarithmic growth at a cell density of 1×10^7 to 2×10^7 cells per ml. Labeling for additional generations produced no changes in the percentage distribution of the label into lipid classes or the specific activity of the lipid extracted, so it was assumed that a steady-state labeling condition was achieved (1, 2).

To examine the rates of synthesis of various lipids, the cells were grown to the logarithmic phase (0.6×10^7 to 2×10^7 cells per ml) in synthetic medium containing inositol and choline supplements as indicated. The cultures were labeled with [*methyl*-^{14}C]methionine (0.5 μCi/ml; specific activity, 57.5 mCi/mmol; New England Nuclear) for 30 min or $^{32}P_i$ (50 μCi/ml) for 15 min.

Lipids were extracted from spheroplasts as described previously (2). Lipids labeled with $^{32}P_i$ were separated by the two-dimensional paper chromatography system of Steiner and Lester (24) on EDTA-treated Whatman SG81 paper. The following solvent systems were used: dimension 1, chloroform–methanol–30% ammonium hydroxide–water, 66:27:3:0.8; and dimension 2, chloroform–methanol–glacial acetic acid–water, 32:4:5:1. ^{14}C-labeled lipids were separated by one-dimensional chromatography by the method of Waechter and Lester (25, 26). Radioactivity was located by autoradiography with Kodak XAR-5 X-ray film. Spots were cut from the chromatogram and counted in a scintillation counter. Lipids were identified by comparison with the migration of the following standards (purchased from GIBCO Laboratories): PI, PS, PE, PC, cardiolipin, and phosphatidic acid.

Immunoprecipitation of inositol-1-phosphate synthase subunit. Inositol-1-phosphate synthase was immunoprecipitated from crude extracts of *opi1* and wild-type strains as previously described (11). The precipitates were electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gels under fully dissociating conditions (21), and the 62,000-dalton subunit of inositol-1-phosphate synthase was visualized by Coomassie blue staining of the gel.

Preparation of cell-free extracts and assay of enzymes. All steps were carried out at 8°C. Cells (ca. 1 g) were washed in distilled water and suspended in 1 ml of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM disodium EDTA, 0.3 M sucrose, and 10 mM 2-mercaptoethanol. The cell suspension was mixed with 1 g of prechilled glass beads (diameter, 0.3 to 0.5 mm) and disrupted in a Mini-Bead-Beater (Biospec Products) by seven 15-per-second bursts, with a 30-s pause between bursts. Glass beads and unbroken cells were removed by centrifugation at $1,500 \times g$ for 5 min. The supernatant (cell-free extract) was used for PS synthase and PI synthase assays. Alternatively, cells were broken with a Braun homogenizer in 5 mM potassium phosphate buffer (pH 7.2) containing 0.2 M sucrose. Whole cells and glass beads were removed by low-speed centrifugation, and the supernatant was used for PE methyltransferase assays.

All enzyme assays were performed at 30°C. PE methyltransferase activity was measured by following the methylation of endogenous PE in the cell-free extract with 1 mM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (specific activity, 10.2 Ci/mmol). Reactions were carried out in the presence of 15 mM potassium phosphate buffer (pH 7.2), 60 mM sucrose, and cell-free extract protein (0.5 mg/ml) in a total volume of 0.5 ml. The reaction was stopped by mixing with 3 ml of chloroform-methanol (2:1, vol/vol) at the indicated time intervals. The mixtures were vortexed and extracted at room temperature for at least 1 h. The organic phase was removed, and a portion of the organic phase was counted by liquid scintillation to determine the total amount of label incorporated into lipid. The remainder of the sample was

dried under nitrogen, and the lipids were separated by paper chromatography in one dimension as described above. The chromatograph was sprayed with En^{3}Hance (New England Nuclear), and the radioactivity was located by autoradiography with Kodak XAR-5 X-ray film. Spots were cut from the chromatogram and counted in a scintillation counter.

PS synthase activity was measured by following the incorporation of 0.5 mM L-[3-^{3}H]serine (10,000 cpm/nmol) into a chloroform-soluble product in the presence of 50 mM Tris-hydrochloride buffer (pH 8.0), 1 mM $MnCl_2$, 0.2 mM CDP-diacylglycerol, 3.2 mM Triton X-100, and cell-free extract (1 mg/ml) in a total volume of 0.1 ml (5). PI synthase activity was assayed in a similar manner (5) by using 0.5 mM *myo*-[2-^{3}H]inositol (10,000 cpm/nmol), 50 mM Tris-hydrochloride buffer (pH 8.0), 2 mM $MnCl_2$, 0.2 mM CDP-diacylglycerol, 2.4 mM Triton X-100, and cell-free extract (1 mg/ml) in a total volume of 0.1 ml. One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per min under the assay conditions described above. The specific activity was defined as the units per milligram of protein. Protein was determined by the method of Bradford (3) with bovine serum albumin as a standard.

RESULTS

Effect of inositol and choline on the phenotype and phospholipid composition of the *opi1* mutant and the wild-type strains. Using the bioassay method of Greenberg et al. (15), we found that the *opi1* mutant strain excreted inositol regardless of whether the medium was supplemented with 1 mM choline. The wild-type yeast cells (*ade5* **a**) did not excrete inositol when grown in either the presence or absence of exogenous choline.

Steady-state labeling of the phospholipids was performed to determine the effects of inositol and choline supplementation on the phospholipid composition of the wild-type (*ade5* **a**) and mutant (*opi1*) yeast strains. The percentage of total label incorporated into each phospholipid was determined (Table 1). As previously reported (16), the wild-type strain (*ade5* **a**) shows an increase in the releative content of PI and a decrease in PS when the cells are grown in the presence of inositol. The addition of choline to the medium appeared to have little effect upon the steady-state composition of wild-type cells (Table 1). In contrast to the wild-type strain, there was little or no fluctuation in the proportion of PI and PS in the *opi1* strain in response to exogenous inositol. The relative rates of synthesis of PS and PI were examined by pulse labeling the cells with ^{32}P. In wild-type cells there was a decrease in the relative rate of PS synthesis and a compensatory increase in the relative rate of PI synthesis when the cells were grown in the presence of exogenous inositol (Table 2). No fluctuation in the relative rates of synthesis of these phospholipids was observed in the mutant *opi1* strain in response to inositol (Table 2). The synthesis of the methylated phospholipids (phosphatidylmonomethylethanolamine [PMME], phosphatidyldimethylethanolamine [PDME], and PC) was also examined by pulse labeling (with [*methyl*-^{14}C]methionine as described above) cells grown in the presence or absence of inositol and choline. A typical separation of *methyl*-^{14}C-labeled phospholipids is shown in Fig. 2. As previously reported (23), the wild-type yeast strain (*ade5* **a**) incorporated a reduced proportion of label from *methyl*-^{14}C-labeled methionine into PC when the growth medium was supplemented with inositol and choline (Table 3; Fig. 2, lane 3). The mutant *opi1* incorporated label into the various methylated phospholipids in approximately the same proportions under all growth conditions (Table 3).

TABLE 1. Phospholipid compositions of wild-type and *opi1* mutant strains grown under various conditions

Strain	Growth supplement		Phospholipid (%)[a]				
	Inositol (μM)	Choline (mM)	PI	PS	PC	PE	Other
Wild type (*ade5* **a**)	0	0	17	12	39	23	9
	50	0	26	8	34	22	10
	50	1	28	8	32	21	11
opi1 mutant	0	0	25	7	33	24	11
	50	0	28	6	32	22	12
	50	1	29	8	35	29	7

[a] Phospholipid compositions were determined by labeling cells to steady state with ^{32}P as described in the text. Numbers given in the body of the table are percentages of total lipid phosphorus determined for each lipid by chromatographic analysis. Total lipid phosphorus was 11.8 to 12.6 nmol/10^7 cells and was not found to be significantly different in either of the strains under any growth condition tested. The category "other" includes the pooled percentages of phosphatidic acid, CDP-diglyceride, cardiolipin, phosphatidylglycerol, and other minor lipid species. The supplements inositol and choline were added at the indicated concentrations to defined medium as described in the text. The data presented here represent the average of at least three experiments. The compositions of the wild-type control strains grown under various conditions are very consistent and are similar to values published previously (1, 2, 16, 22, 23).

Regulation of enzymes involved in phospholipid synthesis in the *opi1* mutant and the wild-type strains. Inositol-1-phosphate synthase is produced by the *opi1* mutant whether or not inositol is present (14, 15). The presence of inositol or choline in the growth medium did not affect the expression of inositol-1-phosphate synthase in the *opi1* mutant (Fig. 3, lanes 1, 2, and 3). In contrast, inositol-1-phosphate synthase was repressed in the wild-type strain (*ade5* **a**) when it was grown in medium containing inositol (Fig. 3, lane 5) whether or not choline was present (Fig. 3, lane 6).

Since it had previously been demonstrated that inositol and choline are required for repression of the phospholipid *N*-methyltransferases in wild-type cells (27), we examined the effect of these precursors on phospholipid methylation in the *opi1* strain. The data shown in Fig. 4 represent the total phospholipid methylation activity assayed in vitro as described above. The total methylation activity is the sum of label incorporated from *S*-adenosylmethionine into the three methylated phospholipids, PMME, PDME, and PC, and represent the composite activity of the three methylation reactions. The in vitro incorporation of label into the individual phospholipids is presented in Table 4. As expected, growth of wild-type cells in the presence of inositol and choline results in a three- to fourfold repression of the total activity (Fig. 4), and the decrease in incorporation of label into PC is reduced more than fivefold (Table 4). The *opi1* cells, in contrast, had the same level of activity whether or not choline and inositol were present (Fig. 4, Table 4). Regardless of the growth condition, the level of phospholipid methylation activity in *opi1* cells was intermediate between the wild-type repressed and derepressed activities (Fig. 4, Table 4).

The activities of PI synthase and PS synthase had not been studied in wild-type cells in response to both inositol and choline. Therefore, these activities were assayed in extracts derived from wild-type and *opi1* mutant cells grown in the presence or absence of the two precursors, inositol and choline, derived from *opi1* mutant and wild-type cells grown

TABLE 2. Phospholipid synthesis in wild-type and *opi1* mutant strains grown under various growth conditions

Strain	Growth supplement		Phospholipid (%)[a]							
	Inositol (μM)	Choline (mM)	PI	PS	PC	PE	PMME	PDME	PA	Other
Wild type (*ade5* a)	0	0	23	26	13	21	1	1	8	7
	50	0	43	14	14	11	3	2	6	7
	50	1	42	16	18	13	2	1	6	2
opi1 mutant	0	0	33	20	10	21	1	1	12	2
	50	0	39	19	8	21	1	1	9	2
	50	1	40	18	9	22	1	1	8	1

[a] Cells were labeled for 15 min with ^{32}P as described in the text. The numbers in the body of the table represent the percentage of total lipid-soluble phosphorus recovered in each lipid. The total incorporation of label in lipid-soluble material was 10 to 16 cpm/10^4 cells and did not appear to vary significantly with respect to strain or growth condition. Growth conditions are identical to those used for the phospholipid composition analysis (Table 1). PA, Phosphatidic acid.

in medium containing no inositol or choline. In wild-type cells, PI synthase activity did not fluctuate in response to either inositol or choline (Table 5). In contrast, PS synthase was regulated in a complex fashion in response to both compounds. Wild-type cells had the highest specific activity of PS synthase when grown in inositol-free medium with or without choline. However, the presence of inositol alone caused about a 40% repression of PS synthase activity. The addition of both choline and inositol to the growth medium resulted in an approximately three- to fourfold repression of PS synthase (Table 5). In contrast, the *opi1* mutant was completely constitutive for PS synthase activity and failed to respond to inositol alone or in combination with choline (Table 5). Furthermore, the specific activity of PS synthase in the *opi1* mutant was elevated some threefold above wild-type levels (Table 5). In contrast, PI synthase activity in the *opi1* mutant was essentially identical to that of wild-type cells and did not fluctuate in response to either precursor (Table 5).

DISCUSSION

To examine regulation of phospholipid synthesis in the *opi1* mutant, it was first necessary to determine the full range of regulation of PI and PS synthesis in wild-type cells. Yeast PI synthase had been purified to near homogeneity and then characterized (13), but its regulation had not been examined. Yeast PS synthase, a product of the *CHO1* gene (22), had been purified to homogeneity and characterized (4). PS synthase had also been shown to be coordinately regulated with the phospholipid methyltransferases in response to choline (6). However, its regulation had not been examined in response to exogenous inositol. The phospholipid *N*-methyltransferases, on the other hand, had not been purified but had already been demonstrated to be repressed by choline only if inositol was present (27).

The results obtained in this study clearly demonstrate that PI synthase is not regulated in response to either inositol or choline in wild-type yeast cells (Table 5). There is little or no fluctuation in the specific activity of this enzyme in response to either inositol or choline. In contrast, PS synthase is regulated in response to both inositol and choline. As previously reported by Carson et al. (6), the addition of choline to inositol-containing medium leads to a reduction (about fourfold) in the specific activity of the enzyme (Table 5). However, the results obtained in this study demonstrate that, in the absence of inositol, choline has no effect on the specific activity of PS synthase (Table 5). Cells grown in the

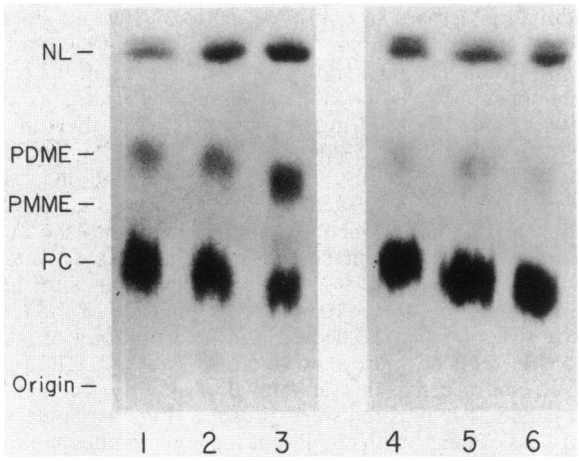

FIG. 2. Synthesis of methylated phospholipids in wild-type (*ade5* a) and *opi1* mutant strains. An autoradiogram of phospholipids labeled in vivo with *methyl*-^{14}C-labeled methionine and separated in one dimension by paper chromatography (as described in the text) is shown. Lanes 1 through 3: Wild-type cells grown in defined medium, with no supplement (lane 1), 50 μM inositol (lane 2), or 50 μM inositol and 1 mM choline (lane 3). Lanes 4 through 6: *opi1* mutant cells grown in defined medium, with no supplement (lane 4), 50 μM inositol (lane 5), or 50 μM inositol and 1 mM choline (lane 6).

TABLE 3. Synthesis of methylated phospholipids in wild-type and *opi1* mutant strains grown under various conditions

Strain	Growth supplement		Lipid fraction (%)[a]				
	Inositol (μM)	Choline (mM)	Origin and SL	PC	PMME	PDME	NL
Wild type (*ade5* a)	0	0	3	64	4	6	23
	50	0	4	59	3	9	25
	50	1	5	44	5	11	35
opi1 mutant	0	0	2	71	1	4	22
	50	0	3	70	2	4	21
	50	1	4	70	2	3	21

[a] The numbers in the table represent the percentage of total lipid-soluble label incorporated into each lipid in a 30-min pulse with *methyl*-^{14}C-labeled methionine. Labeling was carried out as described in the text. Data shown are the average of three experiments. Total incorporation into lipid was 8.4 to 6.5 cpm/10^4 cells for *opi1* mutant cells and did not vary with growth conditions. For wild-type cells, as previously reported (16, 22), total incorporation was 9.2 to 6.8 cpm/10^4 cells for cells grown in the presence or absence of inositol without choline. When choline is present in addition to inositol, the total incorporation is reduced to 3.3 to 2.9 cpm/10^4 cells. In the chromatographic separation used here, sphingolipids (SL) remained near the origin and are so represented. Significant incorporation into neutral lipids (NL) is due to methylation of sterols. The supplements inositol and choline were added as indicated to defined medium as described in the text.

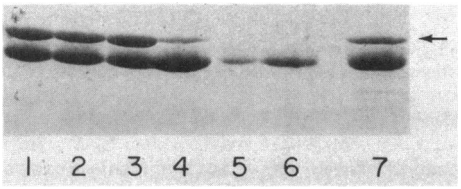

FIG. 3. Immunoprecipitation of inositol-1-phosphate synthase from crude extracts of wild-type (*ade5* a) and *opi1* mutant cells grown under various conditions. The figure shows a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel of the immunoprecipitated 62,000-dalton subunit of enzyme. The position of the enzyme subunit is indicated by an arrow. The protein band migrating directly below the enzyme subunit is immunoglobulin. Immunoprecipitation was carried out as described in the text. Lanes 1 through 3, Immunoprecipitation of crude extracts of *opi1* mutant cells grown without inositol and choline (lane 1), with 50 μM inositol (lane 2), or with 50 μM inositol and 1 mM choline (lane 3); lanes 4 through 6, immunoprecipitation of crude extracts of wild-type cells grown without inositol and choline (lane 4), with 50 μM inositol (lane 5), or with 50 μM inositol and 1 mM choline; lane 7, immunoprecipitation of partially purified inositol-1-phosphate synthase standard.

presence of inositol alone have about 60% of the PS synthase activity found in cells grown in inositol-free medium (plus or minus choline). Thus, inositol alone appears to be slightly repressing for PS synthase. Inositol alone has also recently been shown to have an effect upon expression of the phospholipid *N*-methyltransferases (18; V. Letts and B. Cooperman, personal communication). A consistent twofold stimulation in the specific activity of the phospholipid

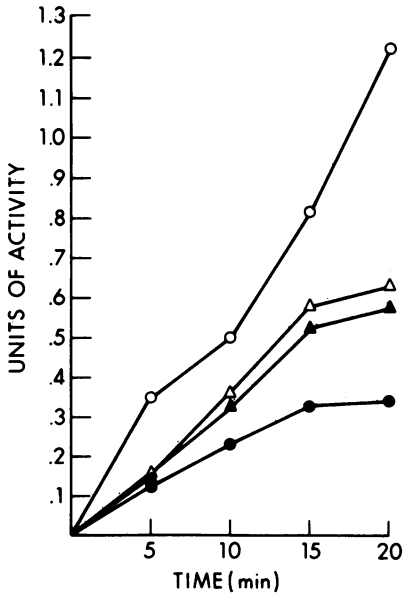

FIG. 4. Methyltransferase activity in *opi1* mutant and wild-type cells grown in the presence and absence of inositol and choline. Total phospholipid methyltransferase activity was assayed in cell-free extracts as described in the text. The reactions were stopped at the time intervals indicated. Units of activity are defined as nanomoles of *S*-adenosylmethionine incorporated into total phospholipid per milligram of protein. Symbols: ○, Wild-type cells (*ade5*a) grown in the absence of supplement; ●, wild-type cells grown in the presence of 50 μM inositol and 1 mM choline; △, *opi1* mutant cells grown in the absence of supplement; ▲, *opi1* mutant cells grown in 50 μM inositol and 1 mM choline.

TABLE 4. Methylation of phospholipids assayed in vitro

Strain	Growth condition (concn of supplements)		Phospholipid (nmol of SAM incorporated per mg of protein)[a]			
	Inositol (μM)	Choline (mM)	PMME	PDME	PC	Total[b]
Wild type (*ade5* a)	0	0	0.09	0.15	1.01	1.25
	75	1	0.07	0.10	0.17	0.34
opi1 mutant	0	0	0.07	0.12	0.44	0.63
	75	1	0.06	0.11	0.39	0.56

[a] The data in the body of the table represent nanomoles of methyl groups from *S*-adenosylmethionine (SAM) incorporated into each phospholipid per milligram of protein in a 20-min assay period. Cell-free extracts were prepared from the *opi1* mutant and wild-type strains as described in the text. The in vitro assay for phospholipid methylation was carried out as described in the text by measuring incorporation of ^{3}H label from *methyl*-^{14}C-labeled SAM into each phospholipid. Each assay was done in duplicate, and the data represent the average of at least two experiments.

[b] Total equals the sum of incorporation of methyl groups from SAM into all three phospholipids (PC, PDME, and PMME).

N-methyltransferases is observed when cells are grown in the presence of inositol (without choline) as compared to cells grown in the absence of inositol (18).

The initial evidence which suggested to us that the *opi1* mutant might have pleiotropic defects in the regulation of phospholipid metabolism was the relatively static phospholipid composition it displayed (Table 1). The phospholipid composition of wild-type cells changes in response to the availability of the various soluble phospholipid precursors, particularly inositol (Table 1; see also references 16 and 17). The relative rates of synthesis of the various phospholipids also shift in response to the changing availability of the precursors in wild-type cells (Tables 2 and 3). In contrast, phospholipid composition (Table 1) and relative rates of synthesis of the various phospholipids (Tables 2 and 3) are virtually unaffected by the presence of inositol or choline in the *opi1* mutant.

It had previously been demonstrated that the *opi1* mutant synthesizes inositol-1-phosphate synthase in a constitutive fashion (14, 15). This enzyme was shown by enzyme assay to be expressed at a level approximately twofold higher in the *opi1* mutant than in wild-type cells, whether or not

TABLE 5. Effects of exogenous inositol and choline on relative activity of PS synthase and PI synthase in wild-type and mutant (*opi1*) yeast strains

Strain	Growth supplement		Synthase sp act (nmol/min/per mg)		Synthase relative activity (%)[a]	
	Inositol (μM)	Choline (mM)	PS	PI	PS	PI
Wild type (*ade5* a)	0	0	0.4	0.3	100	100
	0	1			107	99
	50	0			62	96
	50	1			28	93
opi1 mutant	0	0	1.3	0.4	100	100
	0	1			101	110
	50	0			140	90
	50	1			121	90

[a] Relative activity (%) was calculated by normalizing the specific activities obtained by assay in each strain under the stated growth condition against the specific activity measured in the same strain in the absence of both supplements (i.e., no inositol or choline; specific activities were as shown).

inositol is present (14). The addition of choline has no effect on inositol-1-phosphate synthase expression in the *opi1* mutant, whether or not inositol is present (Fig. 3). PS synthase is also made at a high constitutive level in the *opi1* mutant under all growth conditions (Table 5). In addition, the total phospholipid methylation activity in the *opi1* mutant is intermediate to the wild-type strain repressed level and the level observed in wild-type cells grown in inositol-free medium (Table 4, Fig. 4). Furthermore, phospholipid methyltransferase activity is constant in the *opi1* mutant and fails to be repressed by the combination of inositol and choline in the growth medium (Fig. 4). Thus, one effect of the *opi1* mutation is to alter the steady-state expression of a series of enzymes which are regulated in different ways by inositol. In addition, the *opi1* mutant is pleiotropically constitutive.

The fact that inositol-1-phosphate synthase and PS synthase are constitutively overproduced in the *opi1* mutant is consistent with the hypothesis that the *OPI1* gene product is a negative regulator. Not consistent with this hypothesis is the observation that the activity of the phospholipid *N*-methyltransferases is somewhat lower in the *opi1* mutant than in wild-type derepressed cells. However, in wild-type cells, inositol alone stimulates the phospholipid *N*-methyltransferases (18), whereas it represses inositol-1-phosphate synthase and partially represses PS synthase. Thus, for all of the enzymes the activity in *opi1* cells most resembles the wild-type condition in the absence of inositol. It is possible, therefore, that the *OPI1* gene product functions as part of the cellular machinery involved in "sensing" the presence of inositol and that the *opi1* mutant is, in essence, "blind" to inositol. The fact that the *opi1* mutant fails to respond to the combination of inositol and choline is consistent with this hypothesis, since wild-type cells do not respond to choline unless the presence of inositol is also detected. Detection of inositol by the cell would be expected to be a preliminary step in the regulatory process.

The epistatic relationships of the *opi1* lesion to other pleiotropic regulatory mutations affecting phospholipid metabolism (18) also suggests that the *OPI1* gene product controls an early step in the regulatory process. For example, *opi1 ino2* and *opi1 ino4* double mutants both have the repressed phenotype of the *ino*$^-$ lesions rather than the constitutive phenotype of the *opi1* mutant strain (18). This epistatic relationship suggests that the *OPI1* gene product controls a process in the regulatory cascade which precedes the positive regulatory function controlled by the *INO2* and *INO4* gene products. Future studies will be directed toward dissecting this complex regulation through analysis of the effects of these and other regulatory mutants upon expression of the coordinately regulated genes, their transcripts, and gene products. These studies will be facilitated by the recent isolation of two of the structural genes (*CHO1* and *INO1*) encoding enzymes (PS synthase and inositol-1-phosphate synthase, respectively) which participate in this coordinate regulation (20, 22).

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