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Yeast Mutant Defective in Phosphatidylcholine Synthesis

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The *Saccharomyces cerevisiae opi3-3* mutant was shown to be defective in the synthesis of phosphatidylcholine via methylation of phosphatidylethanolamine. The *opi3-3* mutant was isolated on the basis of an inositol excretion phenotype and was not auxotrophic for choline. Inositol, but not choline, stimulated growth of the mutant. The *opi3-3* mutation was recessive and was genetically linked to the *ino4* locus. When grown in the absence of exogenous choline, the *opi3-3* mutant had a phospholipid composition consisting of 2 to 3% phosphatidylcholine compared with 40 to 50% in wild-type strains. In addition, the mutant accumulated elevated amounts of two intermediates, phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine. The incorporation of label from [*methyl*-^{14}C]methionine into phosphatidylcholine was reduced 80 to 90% in the mutant compared with wild-type strains. However, label was recovered in the intermediates phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine. The mutant is believed to be defective in the third and possibly the second methylation reaction in the formation of phosphatidylcholine from phosphatidylethanolamine. The first methylation reaction appeared to be occurring at normal or even elevated levels. Based upon incorporation of choline into phosphatidylcholine, it is concluded that the *opi3-3* mutant has no defect in the synthesis of phosphatidylcholine from exogenous choline. Furthermore, phosphatidylcholine represents over 25% of the phospholipid composition of the mutant when it is grown in the presence of exogenous choline.

Phosphatidylcholine (PC) is made in yeasts, as in other eucaryotes, by one of two alternative pathways (Fig. 1). De novo synthesis of PC in yeasts occurs in the membrane via three sequential methylations of phosphatidylethanolamine (PE) (21, 22) (Fig. 1, reaction series 1). Alternatively, if exogenous choline is present, yeasts can synthesize PC directly via the pathway first described by Kennedy and Weiss (15) (Fig. 1, reaction series 2). *Neurospora* mutants defective in the formation of PC via methylation of PE were identified among mutants selected as choline auxotrophs (3, 13, 18). However, *Saccharomyces cerevisiae* mutants similar to the *Neurospora* mutants have not been described. The mutations of the majority of choline-auxotrophic yeast mutants isolated are allelic to the *cho1* locus (1, 2, 14, 16) and are defective in the synthesis of phosphatidylserine (PS). The yeast *cho1* mutants have an auxotrophic requirement satisfied by ethanolamine or choline, which the cells incorporate directly into phospholipid, bypassing PS as an intermediate (1, 14, 16).

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Since very little or no PS is synthesized by the *cho1* mutants under any circumstance, it can be concluded that this lipid is largely expendable in the formation of functional membranes in yeasts (2, 14, 16). In addition, when *cho1* cells are grown in the presence of exogenous choline, PC is formed, bypassing both PS and PE as intermediates (Fig. 1). Under such circumstances, the phospholipid composition of *cho1* mutants is very reduced in PE content and consists largely of phosphatidylinositol (PI) and PC (2). The fact that vegetatively grown yeast cells can tolerate extreme alterations in phospholipid composition such as those observed in *cho1* mutants may be the explanation for the failure to detect mutants defective in methylation of PE among yeast choline auxotrophs.

Indeed, the *opi3-3* mutant, which is the subject of this report, is defective in the synthesis of PC via methylation of PE. Similar to the *Neurospora* choline mutants (3, 18), which it resembles in its biochemical defect, the *opi3-3* mutant synthesizes very little PC in the absence of exogenous choline. But although the *Neurospora* mutants require exogenous choline for growth, the *S. cerevisiae op3-3* mutant is not a

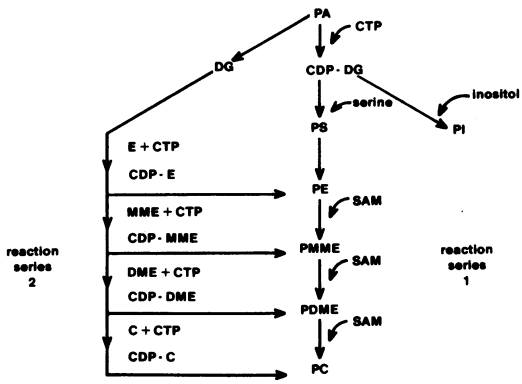

FIG. 1. Pathways for phospholipid synthesis in yeasts. Steiner and Lester (19) detected most of these reactions in vitro in isolated yeast membranes. Waechter and Lester (21, 22) reported the synthesis of PC via methylation of PE in yeast membrane (reaction series 1). Kennedy and Weiss (15) described the formation of PE, PC, and the intermediates PMME and PDME via the reactions depicted in reaction series 2. Abbreviations: E, ethanolamine; MME, monomethylethanolamine; DME, dimethylethanolamine; C, choline; CDP-DG, CDP-diglyceride; SAM, *S*-adenosylmethionine; PA, phosphatidic acid.

choline auxotroph. The *opi3-3* mutant was isolated serendipitously as an inositol-excreting strain in a mutant selection procedure designed to detect mutants with altered regulation of the enzyme inositol 1-phosphate synthase (8, 9). This enzyme is the major biosynthetic enzyme in inositol biosynthesis and in yeasts is repressed over 50-fold in the presence of exogenous inositol (4, 7). In the original screening for inositol-overproducing strains, five independent mutants representing four loci were identified. Four of the mutants representing three loci (*opi1*, *opi2*, and *opi4*) were found to have mutations which conferred the property of constitutive synthesis of inositol 1-phosphate synthase (9). However, the fifth mutant, carrying the *opi3-3* mutation, was not constitutive for inositol 1-phosphate synthase (9). This mutant was tentatively identified as having a defect in phospholipid metabolism since it appeared to accumulate intermediates in the synthesis of PC (10). We present evidence here that demonstrates that the *opi3* mutant has a specific defect in the synthesis of PC. The relationship between this defect and the phenotype of inositol excretion is discussed.

MATERIALS AND METHODS

Strains and detection of the Opi phenotype. The isolation of the *opi3-3* mutant has been previously described (9). This mutant is isolated on the basis of inositol overproduction and excretion. The inositol overproduction (Opi^-) phenotype is detected in a plate assay which involves growing the Opi^- strain on inositol-deficient complete medium (described below) on agar plates for several days (preincubation period). The plates are sprayed with a lawn of diploid strain AID (*ino1-13/ino1-13 ade1/ade1* a/α) as previously described (8, 9). The AID detector strain is an inositol auxotroph and does not grow on inositol-deficient medium. Growth of this strain is only observed in response to inositol excreted from the Opi^- strain. The detector strain grows in a characteristic halo around the excreting strain. Since the strain is also *ade1* homozygous, the halo is red and can be easily distinguished from the growth of the excreting strain. The size of the halo is variable, from about 1 mm to about 1 cm in radius, depending upon how far the inositol has diffused into the agar. The diffusion pattern depends upon a number of variables, all relating to the growth of the Opi^- strain. These variables include the following: relative overproduction of inositol by a particular Opi^- strain, overall growth rate of the strain, time of preincubation (before exposure to the detector strain), and incubation temperature (lower incubation temperature, i.e., 23 versus 30°C, produces smaller halo). Thus, the halo size is variable among Opi^- mutants and may even vary among different strains bearing the same Opi^- mutation. In the crosses performed in this study, in which detection of the Opi^- phenotype was carried out, the conditions routinely used were as follows. One to four evenly spaced colonies, usually derived from a single tetrad, were replica plated onto inositol-deficient medium. This plate was incubated for 2 days at 30°C and was then sprayed with a suspension of the detector strain in sterile distilled water. The plates were incubated for 3 to 5 additional days at 30°C and examined daily for the appearance of the halo.

The two-wild type strains, SHID5C (*ade6 ural*α) and *ade5a* are Opi^+ (fail to overproduce inositol) by the above assay. These two laboratory strains are similar to each other in all of the phospholipid biosynthetic activities examined here and have been used in crosses with the *opi3-3* strain. Both strains have been used as parental strains in various mutant isolation procedures (4, 5, 9, 14).

Media and growth conditions. Strains were maintained on YEPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar). Respiratory sufficiency was tested on YPGE plates with 2% glycerol and 2% ethanol in place of glucose. Synthetic complete medium cointained the following: 2% glucose; 0.67% Difco yeast nitrogen base without amino acids; the amino acids (in milligrams per liter) lysine (20), arginine (10), leucine (10), methionine (10), threonine (60), tryptophan (10), histidine (10), adenine (10), uracil (10), and myo-inositol (3, to total 5 mg/liter with the inositol contained by yeast nitrogen base); and either 1 mM choline, 1 mM ethanolamine, 1 mM serine, or 1 mM *N,N*-dimethylethanolamine (all from Sigma Chemical Co.) or 1 mM *N*-methylethanolamine (from Eastman Chemical Co.). Auxotrophic markers were scored on media lacking a single component of the complete medium. Inositol-less medium was prepared with Difco vitamin-free yeast base as described by Culbertson and Henry (6).

Liquid cultures to be used in labeling and growth studies were pregrown overnight at 30°C in medium containing the various supplements to a density of 1×10^6 to 2×10^6 cells per ml. The optical density of cultures was determined in a Klett spectrophotometer

with a red filter, and the number of cells per milliliter was determined by counting under the microscope on a hemocytometer.

Lipid extraction and separation. Lipids were extracted from spheroplasts as previously described (1, 2). Spheroplasts were prepared by harvesting cells by centrifugation, washing twice with water, and suspending at a density of 5×10^7 to 1×10^8 cells per ml in a digestion mixture consisting of the following: 1.2 M glycerol, 100 mM sodium thioglycolate, 50 mM Tris-SO_4 (pH 7.5), and 0.5 mg of Zymolyase 5000 (Kirin Breweries, Takasaki, Gumma, Japan) per ml. This mixture was devised to address difficulties in cell wall removal after periods of starvation for lipid precursors. Unusually high osmotic support and a sulfhydryl-reducing agent are required to remove cell walls in some circumstances and do not appear to produce damage to normal cells. After 15 min of incubation at 25°C, less than 1% osmotically insensitive whole cells remained, and all spheroplasts were maintained intact. Spheroplasts were separated from the digestion mixture by centrifugation at 1,000 × *g* for 5 min. The supernatant fluid was drawn off as completely as possible because the presence of glycerol in the subsequent lipid extracts distorted the chromatographic separation. Spheroplast pellets were suspended in chloroform-methanol (2:1) for 1 h and washed with 0.2 volume of water. The organic phase was drawn out, and a sample was counted in a scintillation counter to determine total labeled lipid. The remainder was dried under nitrogen and suspended in a small volume of chloroform-methanol for chromatography. The method of lipid extraction used resulted in rapid and efficient extraction of the major yeast phospholipids. The method did not efficiently extract several polar lipids, including the major yeast sphingolipids, which are extractable by other methods (20). The residual polar phospholipids remained close to the origin of the chromatography system and were not resolved. Since they are not of primary interest in this analysis, they were simply tabulated together and are listed as polar lipids in the composition analysis. They include CDP-diglyceride and residual sphingolipids (1, 2).

Lipids labeled with ^{32}P were separated by the two-dimensional paper chromatography system of Steiner and Lester (19, 20), with EDTA-treated Whatman SG81 paper, 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 (21, 22). Radioactivity was located by autoradiography with Kodak X-ray film (Kodak XAR-5). Spots were cut from the chromatogram and counted in a scintillation counter. In some cases, chromatograms were sprayed with ninhydrin or acid molybdate, followed by UV irradiation. Lipids were identified by comparison with the migration of the following standards (purchased from GIBCO Laboratories): PI, PS, PE, PC, cardiolipin, and phosphatidic acid.

Lipid composition analysis. Cells were labeled with $^{32}P_i$ (New England Nuclear Corp.) in synthetic complete medium supplemented as indicated. 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. The cells were 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 change in the percentage distribution of the label into lipid classes or in the specific activity of the lipid extracted, so it is assumed that a steady-state labeling condition was achieved (1, 2).

Pulse-labeling conditions. Cells were grown to the logarithmic phase (0.6×10^7 to 2×10^7 cells per ml) in synthetic complete medium supplemented with 75 μM inositol. The cells were harvested, washed thoroughly with medium lacking inositol, and suspended in fresh medium containing the supplements shown for each experiment. The cultures were incubated at 30°C for 2 h with shaking before labeling with $^{32}P_i$ (50 μCi/ml) for 20 min.

For [^{14}C]methionine labeling studies, cultures were pregrown as described above and then incubated at 30°C for 2 h in synthetic complete medium lacking methionine. L-Methionine (15 μg/ml) and [*methyl*-^{14}C]methionine (0.5 μCi/ml; specific activity, 57.5 mCi/mmol; New England Nuclear) were added, and the cultures were incubated at 30°C for a further 30 min. For [^{14}C]ethanolamine or [^{14}C]choline labeling, cells were cultured overnight in medium supplemented with 0.5 mM ethanolamine or choline, respectively. Label was added at a final specific activity of 0.3 μCi/mmol to logarithmically growing cultures for a 30-min pulse. Incorporation of label into lipid was standardized in each case on the basis of cell number.

RESULTS

Growth characteristics of the *opi3-3* mutant. The *opi3-3* strain was grown in the presence and absence of inositol, ethanolamine, and choline. The growth rate was unaffected by the addition of ethanolamine or choline (Fig. 2). However, growth was measurably stimulated by the addition of inositol. In the absence of inositol, with or without added choline or ethanolamine, the mutant grew with a doubling time of 4.5 to 5 h, whereas in the presence of inositol, it grew with a generation time of 2.5 to 3 h (Fig. 2).

Linkage of the *opi-3* mutation to the *ino4* locus. Previously, we reported that the *opi3-3* mutation is unlinked to the *opi1*, *opi2*, and *opi4* loci, all of which are unlinked to each other and unlinked to the *ino1*, *ino2*, and *ino4* loci (9). We have reexamined the linkage of *opi3-3* to loci *ino1*, *ino2*, and *ino4* by performing tetrad analysis of pairwise crosses of *ino1*, *ino2*, or *ino4* mutants to *opi3-3* strains.

There are several technical difficulties inherent in performing analyses of crosses in which the inositol overproducer (Opi$^-$) phenotype must be scored. Occasionally a weak excretor strain may escape detection by the plate assay described above or may be positive in one test and negative in subsequent tests. In addition to these difficulties, Ino$^-$ × Opi$^-$ crosses pose one additional problem since the Opi$^-$ phenotype can only be scored in an Ino$^+$ genetic background. Therefore, Ino$^-$ spore colonies in such

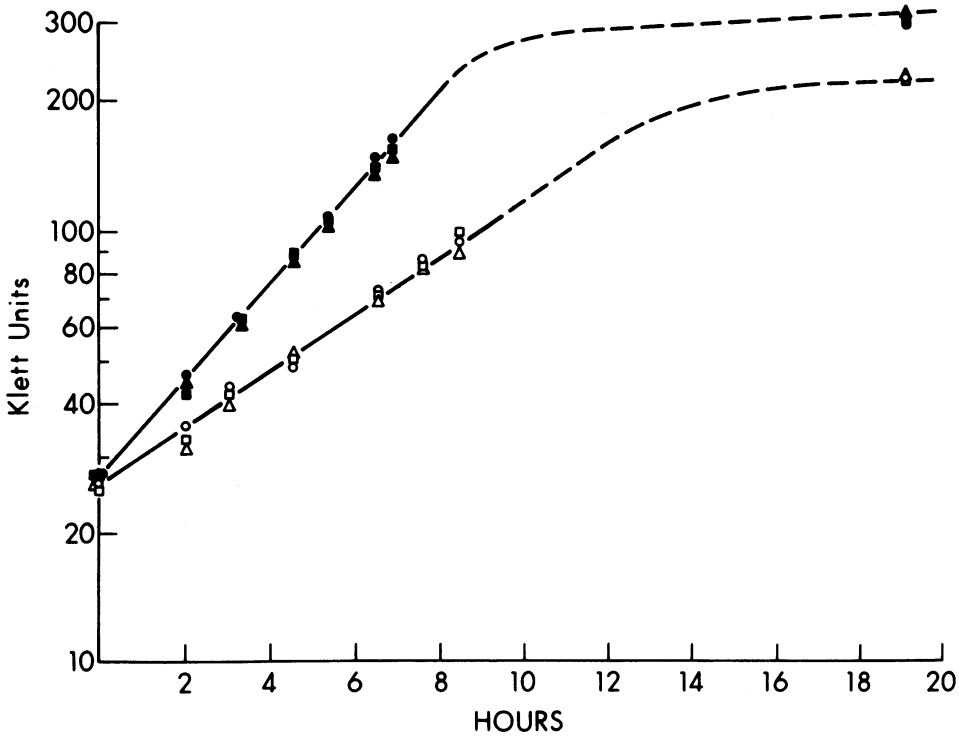

FIG. 2. Growth of the *opi3-3* mutant on inositol, choline, and ethanolamine. Cells were grown in the presence of 75 μM inositol (●), 75 μM inositol and 1 mM choline (■), or 75 μM inositol and 1 mM ethanolamine (▲), or in the absence of all three supplements (○), in the presence of 1 mM choline only (□), or in the presence of 1 mM ethanolamine only (△).

crosses may be either Ino$^-$ Opi$^-$ or Ino$^-$ Opi$^+$. In such crosses tetratype asci are identified by the appearance of one Ino$^+$ Opi+ (inositol nonexcretor) colony, whereas nonparental ditype asci are characterized by the appearance of two such colonies. However, since the reciprocal (Ino$^-$ Opi$^-$) class is hard to identify, it is difficult to distinguish reciprocal recombination from gene conversion. Furthermore, occasional Ino$^+$ Opi$^-$ colonies fail to excrete inositol at a detectable level and may be scored as Ino$^+$ Opi$^+$ recombinants. Even with these reservations, the tetrad analysis given in Table 1 allows the following conclusions to be drawn. The *opi3-3* mutation is unlinked to loci *ino1* and *ino2* and is tightly linked to locus *ino4*. In the 55 tetrads examined from *ino4* × *opi3* crosses, no nonparental ditype and only six tetratype asci were observed. Based upon these data, the *opi3-3* mutation is estimated to be 5.5% recombination units from *ino4*. Considering the reservations stated above, this figure probably represents a considerable overestimate of the frequency of recombination.

The *opi3-3* inositol excretion phenotype is recessive as tested by the red halo assay. In other words Opi$^+$/*opi3-3* diploids are nonexcretors. The biochemical defect is also recessive as discussed below.

Analysis of phospholipid synthesis in the *opi3-3* mutant. The *opi3-3* mutant accumulated abnormal levels of intermediates in the synthesis of PC (9, 10). In the present study, we have examined in detail the synthesis of PC and other phospholipids in the *opi3-3* mutant. We determined the phospholipid composition of the *opi3-3* strains grown in the presence and absence of inositol, ethanolamine, and choline. Several features of the phospholipid composition of the *opi3* strain are different from those of the control wild-type strain (Table 2). Most striking is the deficiency of PC and, to a lesser degree, PE; and the excessive accumulation of the intermediate phosphatidylmonomethylethanolamine (PMME) and, to a lesser degree, phosphatidyldimethylethanolamine (PDME). These lipids are present only at trace levels in the control strain. The only condition under which a substantial amount of PC was synthesized by the *opi3-3* mutant was when exogenous choline was supplied in the

TABLE 1. Crosses of *opi3-3* with *ino* mutants[a]

Cross	PD (2 *ino*$^-$:2 *opi*$^-$)	NPD (2 *ino*$^-$:2+)	TT (2 *ino*$^-$:1 *opi*$^-$:1+)
ino1-13 (*lys2α*) × *opi3-3* (*ade5a*)	3	1	8
ino2-21 (*ade5a*) × *opi3-3* (*lys2α*)	1	5	2
ino4-38 (*lys2α*) × *opi3-3* (*ade5a*)	14	0	2
ino4-40 (*lys2α*) × *opi3-3* (*ade5a*)	20	0	0
ino4-39 (*lys2α*) × *opi3-3* (*ade5a*)	15	0	4
Pooled data (*ino4* × *opi3*)[b]	49	0	6

[a] Abbreviations: PD, parental ditype; NPD, nonparental ditype, TT, tetratype.

[b] Total pooled *ino4* × *opi3* recombination frequency = TT/2(total asci) = 6/110 = 5.5%.

growth medium (Table 2). Even under these conditions, the relative proportion of PC was lower than in the control strain.

Finally, the phospholipid composition analysis reveals another more subtle difference between the *opi3* strain and the wild-type control. The wild-type phospholipid composition varied dramatically in response to exogenous inositol. In the absence of supplied inositol, the total percentage of PI dropped to about 11% in the wild-type strain, and the relative proportion of PS and PC increased. In contrast, the *opi3-3* strain made a relatively high proportion of PI (20 to 24%) even in the absence of inositol. The relatively high proportion of PI synthesis in the *opi3-3* strain in the absence of supplied inositol is consistent with the inositol excretion phenotype and suggests that inositol is overproduced relative to general metabolism. However, the observation of enhanced growth of the *opi3-3* strain in response to inositol is more difficult to reconcile with these observations and is discussed below.

Pulse-labeling of phospholipids with $^{32}P_i$. The phospholipids of *opi3-3* and wild-type strains were labeled for 30 min with $^{32}P_i$ under the growth conditions employed in the phospholipid composition analysis. However, the shorter labeling period employed in this experiment resulted in rapid labeling of lipids toward the beginning of the pathways illustrated in Fig. 1. Hence, PI and PS, for example, were relatively more heavily labeled in these short-term labeling experiments than in the composition studies, whereas PC was relatively less heavily labeled (Table 3). The *opi3-3* strain exhibited reduced synthesis of PC, except in the presence of exogenous choline (Fig. 3) when the relative rate of PC synthesis was actually elevated relative to the control strain (Table 3). In contrast, steady-state labeling (Table 2) revealed somewhat lower overall levels of PC compared with those in the wild type, even when the mutant was grown in the presence of choline. This apparent discrepancy may result from the fact that PC is not rapidly labeled with $^{32}P_i$ when it is being synthesized via methylation of PE. Thus, the overall contribution of the methylation pathway to total PC synthesis in the wild-type cells is not reflected in the pulse-labeling experiments. Alternatively, or in addition, it is possible that the mutant also has an elevated rate of PC turnover. The *opi3-3* strain also synthesized excessive quantities of the two intermediates PMME and

TABLE 2. Phospholipid composition of the *opi3-3* mutant

Organism	Growth supplement	Phospholipids (%)[a]							
		PI	PS	PC	PE	PMME	PDME	PA	Other
opi3-3 mutant	None	21.0	3.7	1.9	7.3	48.4	10.6	2.3	4.8
	Ethanolamine	20.1	5.4	0.9	12.9	43.2	9.4	4.0	4.1
	Choline	24.0	5.7	26.6	9.9	23.5	3.0	3.5	3.8
	Inositol	30.6	2.8	3.0	5.3	34.9	12.5	2.7	7.6
	Inositol + ethanolamine	24.9	3.5	1.3	21.3	31.5	6.1	3.8	7.6
	Inositol + choline	26.4	3.8	28.1	15.4	16.7	2.7	4.0	2.9
Wild type (*ade5a*)	None	10.9	8.5	53.2	15.5	tr	tr	2.6	9.3
	Choline	10.7	9.4	50.5	17.8	tr	tr	3.9	7.7
	Inositol	29.1	5.4	43.2	13.7	tr	tr	3.4	5.2
	Inositol + choline	27.9	4.4	44.9	16.6	tr	tr	3.3	3.0

[a] The numbers in the body of the table are percentages of total lipid extractable phosphorous detected in each phospholipid after steady-state labeling of the cells with $^{32}P_i$ as described in the text. Total lipid phosphorous varied from 11 to 13.5 nmol/10^7 cells and was not demonstrably influenced by the presence of supplements. PA, Phosphatidic acid; other, all other phospholipids including polar lipids remaining near the origin (Fig. 3) and phosphatidylglycerol and cardiolipin; tr, lipids present at trace levels only.

TABLE 3. Pulse-labeling of *opi3-3* lipids with $^{32}P_i$

Organism	Growth supplement	Total cpm/10^4 cells	Phospholipids (%)[a]							
			PI	PS	PC	PE	PMME	PDME	PA	Other
opi3-3 mutant	None	2.6	26.5	24.9	2.1	16.7	14.9	2.5	8.6	3.8
	Ethanolamine	5.5	18.3	19.5	2.0	35.6	11.9	2.7	8.6	1.4
	Choline	6.5	20.7	21.2	25.3	8.9	6.5	0.9	8.6	7.9
	Inositol	8.7	47.0	13.6	2.7	15.0	9.5	2.5	2.3	7.4
	Inositol + ethanolamine	5.7	39.0	8.3	3.8	31.7	5.7	2.3	2.8	6.4
	Inositol + choline	7.7	38.2	15.3	20.2	7.8	3.2	0.5	2.9	11.9
Wild type (*ade6 ura1α*)	None	7.7	26.6	32.3	15.4	13.3	tr	tr	5.6	6.8
	Choline	6.9	28.2	33.7	12.3	10.6	tr	tr	4.9	10.3
	Inositol	16.1	57.4	15.3	8.3	9.6	tr	tr	3.3	6.1
	Inositol + choline	23.1	51.5	18.0	16.6	7.5	tr	tr	3.2	3.2

[a] The numbers are percentages of total lipid extractable ^{32}P after labeling the cells for 20 min as described in the text. Phospholipid designations and abbreviations are as in footnote *a* of Table 2.

PDME, which were found in only trace quantities in the wild type. In the wild-type strain, the proportions of PI and PS varied in response to exogenous inositol. In the wild type, the rate of synthesis of PI was elevated nearly twofold, and the rate of synthesis of PS decreased by a similar proportion in the presence of exogenous inositol. The *opi3-3* strain showed a much less dramatic shift in the relative proportions of PI and PS in response to exogenous inositol.

Labeling with [^{14}C]ethanolamine and [^{14}C]choline. The cells of the *opi3-3* and the wild-type strains were labeled with the phospholipid precursors [^{14}C]ethanolamine and [^{14}C]choline (Table 4). The total uptake of the two labeled precursors into lipid was, if anything, slightly elevated in the *opi3-3* strain compared with that in the wild type. In both strains approximately 98% of the lipid-soluble label derived from [^{14}C]choline was recovered in PC. However, the pattern of incorporation of [^{14}C]ethanolamine into lipid differed in the two strains. In the 30-min labeling period employed, the wild-type strain accumulated approximately 75% of the lipid-soluble label from [^{14}C]ethanolamine into PE. Approximately 10% of the label was recovered in PC, and smaller amounts were detected in PMME and PDME. In the *opi3-3* strain, in contrast, a substantial proportion of the label was recovered from the intermediate PMME, but only 1.5% of the label reached PC.

Labeling of phospholipids with [*methyl*-^{14}C]methionine. The synthesis of PC from PE involves three sequential methylations with *S*-adenosylmethionine as the methyl donor (Fig. 1, reaction series 1). In the wild-type cells, the three phospholipids PMME, PDME, and PC were rapidly labeled in the presence of [*methyl*-^{14}C]methionine (Table 5). The wild-type cells incorporated 50% or more of the label into PC. A smaller percentage was recovered from PDME and PMME. The ^{14}C recovered in neutral lipids presumably was due to methylation of sterols (17). In the *opi3-3* strain the total label incorporated into lipid from [*methyl*-^{14}C]methionine was quite comparable to that of the wild type on a per cell basis (Table 5). However, the distribution of the label was very different. The most

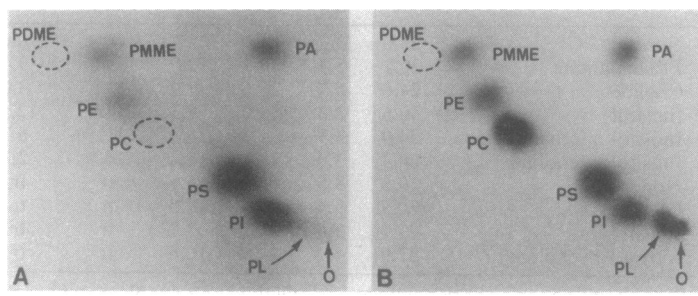

FIG. 3. Lipids of the *opi3-3* mutant. Autoradiograms of ^{32}P-pulse-labeled phospholipids separated by two-dimensional paper chromatography. A, Cells of the *opi3-3* strain were grown in the presence of 75 μM inositol during the labeling period. B, Cells of the *opi3-3* strain were grown in the presence of 75 μM inositol and 1 mM choline. The label appears in PC only in the cells grown in choline-supplemented medium. PL, Polar lipids; PA, phosphatidic acid.

TABLE 4. Incorporation of [^{14}C]ethanolamine and [^{14}C]choline into lipids[a]

Lipid	% [^{14}C]ethanolamine		% [^{14}C]choline	
	ade5	*opi3*	*ade5*	*opi3*
PC	10.1	1.5	97.8	98.1
PE	74.9	48.7	tr	tr
PMME	8.3	42.4	tr	tr
PDME	6.7	7.4	tr	tr
NL	tr	tr	2.2	1.9

[a] The numbers in the body of the table represent the percentage of total lipid extractable ^{14}C detected in each phospholipid after labeling the cells with [^{14}C]ethanolamine or [^{14}C]choline as described in the text. NL, Neutral lipids, including methylated sterols which run at the solvent front during chromatography; tr, trace levels only. The total counts per minute per 10^4 cells were as follows: for *ade5*, 11.0 cpm of [^{14}C]ethanolamine and 21.3 cpm of [^{14}C]choline; for *opi3*, 22.5 cpm of [^{14}C]ethanolamine and 38.0 cpm of [^{14}C]choline.

TABLE 6. Phospholipid methylation in *opi3* haploid and heterozygous diploid[a]

Lipid	% Incorporation of [*methyl*-^{14}C]methionine		
	*ade5***a** (haploid)	*opi3-3 lys1* (haploid)	*opi3-3 lys1*α × *ade5***a** (heterozygous diploid)
PC	59.4	4.6	60.0
PMME	1.7	24.3	1.9
PDME	10.2	18.2	17.7
NL	26.0	50.5	17.3

[a] The data represent incorporation of label from [*methyl*-^{14}C]methionine into lipid after a 30-min labeling period. The data represent an average of at least four separate experiments. The incorporation into each phospholipid is expressed as a percentage of total lipid label. NL, Neutral lipid.

heavily labeled phospholipid in the *opi3-3* strain was PMME, and PDME was also more heavily labeled than in the control strain. However, in the *opi3-3* strain only 3 to 10% of the label was recovered in PC.

A diploid strain (*OPI3 ade5***a** × *opi3-3 lys1*α) was constructed to determine whether the biochemical deficiency in phospholipid methylation is dominant or recessive (Table 6). The diploid resembled the wild-type parent rather than the *opi3-3* parent. Therefore, the mutation is recessive.

DISCUSSION

The *opi3-3* mutant does not appear to have any defect in the synthesis of PC from exogenous choline (Fig. 1, reaction series 2). Incorporation of [^{14}C]choline into PC was comparable or even slightly elevated in the *opi3-3* mutant compared with the wild type (Table 4). Furthermore, the mutant incorporated $^{32}P_i$ into PC when exogenous choline was present (Tables 2 and 3, Fig. 3). However, when exogenous choline is absent from the growth medium very little $^{32}P_i$ was incorporated into PC in the *opi3-3* mutant (Tables 2 and 3). Indeed, under these conditions, PC represented only a few percent of the total phospholipid composition of the *opi3-3* mutant compared with 40 to 50% in the wild-type yeast (Table 2). These results are consistent with a deficiency in the de novo formation of PC via methylation of PE (PE → PMME → PDME → PC). The accumulation of abnormal levels of the two intermediates, PMME and PDME, in the membranes of the *opi3-3* strain (Tables 2 to 5) provides additional support for this hypothesis. However, the data suggest that the first of the three methylation reactions (PE → PMME) is not affected and, if anything, may be slightly

TABLE 5. Labeling of lipids with [*methyl*-^{14}C]methionine

Organism	Growth supplement	Total cpm/10^4 cells	Phospholipids (%)[a]				
			PC	PDME	PMME	PL	NL
opi3-3 mutant	None	3.2	5.8	24.3	38.2	2.2	28.9
	Ethanolamine	3.2	5.2	19.1	36.9	10.7	27.2
	Choline	2.2	2.9	14.1	34.8	11.3	36.2
	Inositol	7.0	10.0	28.7	27.0	3.0	30.2
	Inositol + ethanolamine	4.8	9.7	19.5	28.0	4.5	37.2
	Inositol + choline	3.3	3.3	11.8	22.4	4.2	56.8
Wild type (*ade6 ura1*)	None	8.7	66.3	7.1	1.4	4.8	19.2
	Ethanolamine	9.5	63.6	8.0	1.2	7.2	18.9
	Choline	6.9	58.8	7.6	1.2	5.7	26.0
	Inositol	6.3	57.9	7.8	1.1	10.9	20.6
	Inositol + ethanolamine	6.4	55.7	8.5	1.2	12.8	20.7
	Inositol + choline	2.9	48.7	5.2	1.0	11.9	31.7

[a] The numbers represent the percentage of total lipid detected in each phospholipid after labeling the cells with [*methyl*-^{14}C]methionine for 30 min as described in the text. PL, Polar lipids; NL, neutral lipids.

elevated in the *opi3-3* mutant. For example, a somewhat elevated rate of synthesis of PMME from PE is suggested by the more rapid metabolism of [^{14}C]ethanolamine (Table 4).

The labeling pattern of each of the lipids in this series of reactions is obviously the result both of relative rate of synthesis and of relative rate of utilization as a precursor in subsequent reactions. Since PMME would normally serve as a precursor for the synthesis of PDME, the excessive accumulation of PMME in the mutant may reflect both slightly more rapid synthesis of PMME and reduced capacity of *opi3-3* cells to carry out the second of the two methylations, namely, PMME → PDME. However, this reaction is carried out at some level of activity, since there is accumulation of the product of the second reaction, i.e., PDME. Certainly, the third reaction (PDME → PC) is defective in the *opi3-3* mutant since very little label reached PC in any of the experiments performed, even though the precursor PDME accumulated. The data support the conclusion that the overall reaction series, PMME → PDME → PC, is quite defective in the *opi3-3* mutant. The question that cannot be resolved in this in vivo analysis of phospholipid synthesis is to what degree defects in each of the last two methylation reactions individually contribute to the overall decrease in PC biosynthesis. It will be necessary to assay the individual reactions in vitro in isolated *opi3-3* membranes. These experiments are in progress.

The *opi3-3* mutant of *S. cerevisiae* is similar in its phospholipid composition to a mutant of *Neurospora* sp., strain 47904, whose properties were described by Crocken and Nyc (3) and Scarborough and Nyc (18). The *Neurospora* mutant 47904 accumulates elevated levels of both PMME and PDME (3), and in vitro studies of isolated microsomal membranes (18) of this mutant showed that it is defective in carrying out the two final methylations (PMME → PDME → PC). Another *Neurospora* mutant, strain 34486, was defective in the first methylation (PE → PMME), but had normal activity in the final two methylations (3, 18). Scarborough and Nyc (18) concluded that the first methylation reaction is carried out by one enzyme which is defective in mutant 34486 and that the last two methylations are carried out by a single enzyme which is defective in mutant 47904. Considerable evidence has accumulated in a variety of eucaryotic organisms (11, 12) to support the hypothesis that the methylation sequence PE → PMME → PDME → PC is carried out by two membrane-associated enzymes, the first reaction being catalysed by one enzyme and the last two reactions catalyzed by a single bifunctional enzyme. The characteristics of the *opi3-3* mutant of *S. cerevisiae* are consistent with a lesion which destroys much, but not all, of the activity of the second enzyme.

While the *S. cerevisiae opi3-3* mutant is quite similar in its biochemical properties to the *Neurospora* mutant 47904, its biological properties are quite different. The *Neurospora* mutants 47904 and 34486 were identified as choline auxotrophs (3, 13, 18), and the growth of the *Neurospora* mutants is substantially stimulated by choline (13, 18). This is not true of the *S. cerevisiae opi3-3* mutant. Exogenous choline has no effect on the growth of the *opi3-3* mutant (Fig. 2). The *opi3-3* mutant grows at a rate similar to that of wild-type strains (2 to 2.5 h) on inositol-supplemented medium at 30°C. Since the *S. cerevisiae opi3-3* mutant makes less than 10% of the PC found in wild-type strains under a variety of conditions (Table 2), it seems clear that *S. cerevisiae* is less sensitive to substitution of the various methylated phospholipids in its membranes than is *Neurospora* sp. This result also explains why efforts to obtain yeast mutants defective in the phospholipid methyltransferases failed when the selection was based upon choline auxotrophy (2, 14, 16).

The identification of the *opi3-3* mutant was based upon excretion of inositol into its growth medium (9). Other mutants having this phenotype (*opi1 opi2 opi4* mutants) (9) are all constitutive for the biosynthetic enzyme inositol 1-phosphate synthase gene product of the *INO1* locus. Mutant *opi3-3* is not constitutive for inositol biosynthesis (9), but qualitative inspective of immunoprecipitates of crude extracts of derepressed cells of the *opi3-3* strain indicate that the enzyme inositol 1-phosphate synthase may be produced in excess compared with wild-type, derepressed cells (L. Klig, unpublished observations). Excessive production of this enzyme by the derepressed cells would explain overproduction and excretion of inositol by the mutant. However, growth of the *opi3-3* mutant is stimulated by exogenous inositol (Fig. 2), despite the apparent excessive production of this metabolite. The growth data are difficult to reconcile with the other characteristics of the mutant, but it may be that the alterations in PI biosynthesis observed in the mutant influence its requirement for inositol. The mutant produces a high proportion of PI even in the absence of inositol (Tables 2 and 3). Elevated levels of PI have also been observed in the *cho1* mutant of *S. cerevisiae*, which requires ethanolamine or choline for growth and which is defective in PS synthesis (1, 2). The *cho1* mutant, like the *opi3-3* mutant, also excretes inositol, and PI synthesis has been demonstrated to be tightly coupled to PC synthesis in the *cho1* mutant (2, 10).

Thus, changes in the metabolism of PC or its precursors are known to result in perturbations

of metabolism of inositol or PI (or both) in several yeast mutants. Striking examples of simultaneous alterations in inositol metabolism and PC synthesis occur also in the *S. cerevisiae ino2* and *ino4* mutants (10). The *ino4* locus was shown in this study to be linked to *opi3-3*. The linkage data (Table 1) place this locus within five recombination units of the *opi3-3* mutation. However, since these data, as discussed previously, may be an overestimate of the recombination frequency, the *ino4* locus and the *opi3-3* may be considerably closer. Indeed, the possibility that the two types of mutations (*opi3* and *ino4*) represent different lesions in the same gene cannot be ruled out on the basis of these data. Like the *opi3* mutant, the *ino4* mutants are defective in PC biosynthesis and accumulate some PMME and some PDME, but are not auxotrophic for choline (10; and Loewy and Henry, manuscript in preparation). Unlike the *opi3-3* mutant, however, the *ino4* mutants accumulate primarily PE. The *ino4* mutants also, unlike the *opi3-3* mutant, require inositol due to a failure to synthesize the enzyme inositol 1-phosphate synthase, gene product of the *INO1* locus. We believe that the pleiotropic phenotypes of the *ino4* mutants and the *opi3* mutant are reflections of an intricate coordination of the synthesis of these various phospholipids and their precursors. This coordinate regulation is presently under investigation.

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