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Saccharomyces cerevisiae Mutant with a Partial Defect in the Synthesis of CDP-Diacylglycerol and Altered Regulation of Phospholipid Biosynthesis†

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A *Saccharomyces cerevisiae* mutant (*cdg1* mutation) was isolated on the basis of an inositol excretion phenotype and exhibited pleiotropic deficiencies in phospholipid biosynthesis. Genetic analysis of the mutant confirmed that the *cdg1* mutation represents a new genetic locus and that a defect in a single gene was responsible for the Cdg1 phenotype. CDP-diacylglycerol synthase activity in mutant haploid cells was 25% of the wild-type derepressed level. Biochemical and immunoblot analyses revealed that the defect in CDP-diacylglycerol synthase activity in the *cdg1* mutant was due to a reduced level of the CDP-diacylglycerol synthase M_r-56,000 subunit rather than to an alteration in the enzymological properties of the enzyme. This defect resulted in a reduced rate of CDP-diacylglycerol synthesis, an elevated phosphatidate content, and alterations in overall phospholipid synthesis. Unlike wild-type cells, CDP-diacylglycerol synthase was not regulated in response to water-soluble phospholipid precursors. The *cdg1* lesion also caused constitutive expression of inositol-1-phosphate synthase and elevated phosphatidylserine synthase. Phosphatidylinositol synthase was not affected in the *cdg1* mutant.

The liponucleotide CDP-diacylglycerol (CDP-DG) is an important branch point intermediate in the primary route of biosynthesis of the major phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) in the unicellular eucaryote *Saccharomyces cerevisiae* (19, 39). CDP-DG is the direct source of the phosphatidyl moiety for the synthesis of PI and PS (19, 39). PS is then utilized for the synthesis of PE and PC (19, 39). Consequently, the synthesis of CDP-DG is likely to play an important regulatory role in the coordinate regulation between PI and PC biosynthesis (21, 33). When PS synthesis is blocked, *S. cerevisiae* synthesizes PE and PC from diacylglycerol (11, 29) when supplemented with ethanolamine or choline (1, 2, 31, 37). Diacylglycerol, like CDP-DG, is derived from phosphatidate (PA) and is the precursor of triacylglycerols (11, 19). Therefore, the regulation of CDP-DG synthesis from PA is likely to influence the overall regulation controlling the proportional synthesis of phospholipids and triacylglycerols (28).

In this report, we describe the isolation of a mutant that is simultaneously defective in the synthesis of CDP-DG and in the overall regulation of phospholipid synthesis. This mutant was detected in the course of screening for *opi* mutants that overproduce inositol and excrete it into the growth medium. Among the inositol-overproducing mutants previously described are mutants defective in various aspects of phospholipid metabolism and regulation. For example, the *opi1* mutant constitutively overproduces inositol-1-phosphate (IP) synthase (16, 30), CDP-DG synthase (25), and PS synthase (30, 38). The *opi3* mutant has a defect in the two final methylation reactions leading to the formation of PC (i.e., phosphatidylmonomethylethanolamine [PMME] → phosphatidyldimethylethanolamine [PDME] → PC), as well as a defect in regulation of inositol biosynthesis (17). The mutant described in this report differs from all of the previously described *S. cerevisiae* mutants defective in phospholipid regulation in that it constitutively expresses low levels of CDP-DG synthase while constitutively overproducing IP synthase and overproducing PS synthase.

MATERIALS AND METHODS

Materials. Growth medium supplies were purchased from Difco Laboratories. Bovine serum albumin, CTP, phospholipids, *myo*-inositol, L-serine, ethanolamine, and choline were purchased from Sigma Chemical Co. Radiochemicals were obtained from ICN Pharmaceuticals, Inc., Amersham Corp., and Dupont-New England Nuclear Corp. Triton X-100 was a gift from the Rohm & Haas Co. Scintillation supplies were purchased from either New England Nuclear Corp. or National Diagnostics. Molecular weight standards, electrophoresis, and immunochemical reagents were purchased from Bio-Rad Laboratories. Unmodified nitrocellulose paper (0.2-μm pore size) was obtained from Schleicher & Schuell, Inc., and Whatman no. 42 filter paper was purchased from Fisher Scientific Co. PA (26) and CDP-DG (8) were prepared from soybean lecithin as described previously.

Yeast strains and mutant isolation. Descriptions and the

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
W303-1A (wild type)	*Mata leu2-3 leu2-112 trpl1-1 can1-100 ura3-1 ade2-1 his3-11 his3-15*	R. Rothstein
ade5 strain (wild type)	*MATα ade5*	12
ade5 strain (wild type)	*MATa ade5*	12
MC13	*MATα ino1-13 lys2 can1*	12
MC21	*MATα ino2-21 lys2*	12
MC39	*MATα ino4-39 lys2*	12
OP1	*MATα opi1-1 lys2*	16
OP2	*MATα opi2-2 lys2*	16
OP3	*MATα opi3-3 lys1*	16
OP4	*MATα opi4-4 lys2*	16
C6B	*MATα cho2-1 lys2*	E. Summers
AID-1	*MATa ade1 ino1-13 lys2* / *MATα ade1 ino1-13 ade5*	16
cdg1 strains	*MATa cdg1 ade5*	This study
	MATα cdg1 ade5	This study
	MATa cdg1 ino1-13	This study
	MATα cdg1 leu2	This study

sources of the strains used in this study are shown in Table 1.

The use of the inositol excretion indicator strain AID-1 to detect the Opi⁻ phenotype was described by Greenberg et al. (16). To detect the Opi⁻ phenotype, colonies were replicated onto agar plates lacking inositol. A lawn of the inositol excretor indicator strain was sprayed over the colonies replicated onto inositol-free plates. Inositol-excreting mutants were identified by the red halo that surrounded them when the excreted inositol permitted the red inositol auxotrophic indicator strain to grow. The *cdg1* mutant described in this report was isolated in a screening of mutagenized colonies for the Opi⁻ phenotype following mutagenesis of the *ade5* strain with ethyl methanesulfonate as previously described (16). In the screening that resulted in the isolation of the *cdg1* mutant, 26 mutants exhibiting an inositol excretion ring were detected in the bioassay. The *cdg1* mutant exhibited the strongest inositol excretion pattern of all the mutants, including the previously described *opi1* mutant (16). Tetrad analysis and other genetic methods were performed as previously described (36).

Growth conditions and preparation of cell extracts. Cultures were maintained on YEPD (1% yeast extract, 2% peptone, 2% glucose) medium. The composition of complete synthetic medium is described elsewhere (12, 20). Diploids were induced to undergo meiosis by starvation for nitrogen on media containing potassium acetate (9.8 g/liter), yeast extract (1.25 g/liter), inositol (4 mg/liter), and glucose (1.0 g/liter). Medium plates contained 2% Bacto-Agar. Auxotrophic markers were scored on a medium lacking a single component of the complete medium.

For growth studies, cells were grown at 28°C in complete synthetic medium containing inositol (50 μM), serine (1 mM), ethanolamine (1 mM), and choline (1 mM) where indicated. Cells were grown to the late exponential phase and harvested by centrifugation (30). Cell extracts were prepared by cell disruption with glass beads (30).

Phospholipid analysis. Pulse-labeling of phospholipids with $^{32}P_i$ (50 μCi/ml) and [*methyl*-^{14}C]methionine (0.5 μCi/ml) and steady-state labeling of phospholipids with $^{32}P_i$ (50 μCi/ml) were performed as described previously (1, 30). The phospholipid composition of cells labeled with $^{32}P_i$ and [*methyl*-^{14}C]methionine were determined by two-dimensional paper chromatography and one-dimensional paper chromatography, respectively (1, 30).

Enzyme assays. All assays were conducted at 30°C for 20 min. CDP-DG synthase (CTP:phosphatidate cytidylyltransferase, EC 2.7.7.41) activity was measured with 50 mM Tris-maleate buffer (pH 6.5), 20 mM $MgCl_2$, 15 mM Triton X-100, 1.0 mM [5-^{3}H]CTP (10,000 cpm/nmol), 0.5 mM PA, and enzyme protein in a total volume of 0.1 ml (5). PI synthase (CDP-diacylglycerol:*myo*-inositol 3-phosphatidyltransferase, EC 2.7.8.11) activity was measured with 50 mM Tris hydrochloride buffer (pH 8.0), 2 mM $MnCl_2$, 2.4 mM Triton X-100, 0.5 mM [2-^{3}H]inositol (10,000 cpm/nmol), 0.2 mM CDP-DG, and enzyme protein in a total volume of 0.1 ml (9). PS synthase (CDP-diacylglycerol:L-serine *O*-phosphatidyltransferase, EC 2.7.8.8) activity was measured with 50 mM Tris hydrochloride buffer (pH 8.0), 1.0 mM $MnCl_2$, 3.2 mM Triton X-100, 0.5 mM [3-^{3}H]serine (10,000 cpm/nmol), 0.2 mM CDP-DG, and enzyme protein in a total volume of 0.1 ml (9). The phospholipid products of each reaction were identified with standards by thin-layer chromatography (5, 9). All reactions were linear with time and protein concentration. 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 units per milligram of protein.

Colony autoradiography. Colonies on YEPD plates were replica printed onto sterile Whatman no. 42 filter paper disks, permeabilized by air drying, and assayed for CDP-DG synthase, PI synthase, and PS synthase activities with radiolabeled substrates as described by Homann and Carman (24). The assay conditions for colony autoradiographic analysis of each enzyme were the same as those described above for the in vitro assays except that phospholipid substrates were omitted from the reaction mixtures. PA and CDP-DG were omitted in the assays to assess the activities of the enzymes under conditions which reflect the in vivo concentrations of these substrates. Halos present on the autoradiograms, resulting from enzyme activities of colonies on replica prints, were used to identify mutant colonies. The radiolabeled phospholipid products synthesized by the colonies were extracted and identified by using the appropriate standard phospholipids (24).

Purification of enzymes and preparation of antibodies. Purified preparations of CDP-DG synthase (27), PS synthase (3), PI synthase (14), and IP synthase (13) were prepared as described previously. Polyclonal antibodies were prepared against purified preparations of CDP-DG synthase (27), PS synthase (38), PI synthase (15), and IP synthase (13) as previously described.

Electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32) was performed with 10% slab gels as described previously (14). Proteins were transferred electrophoretically to unmodified nitrocellulose papers (7, 18) as described by Poole et al. (38). The nitrocellulose blots were probed with a 1:500 dilution of CDP-DG antiserum, a 1:500 dilution of PS synthase antiserum, a 1:50 dilution of PI synthase antiserum, and a 1:5,000 dilution of IP antiserum. Proteins were detected on the blots by using a 1:3,000 dilution of goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (40) as described by Poole et al. (38). Immunoblot signals were in the linear range of detectability.

Protein determination. Protein concentration was deter-

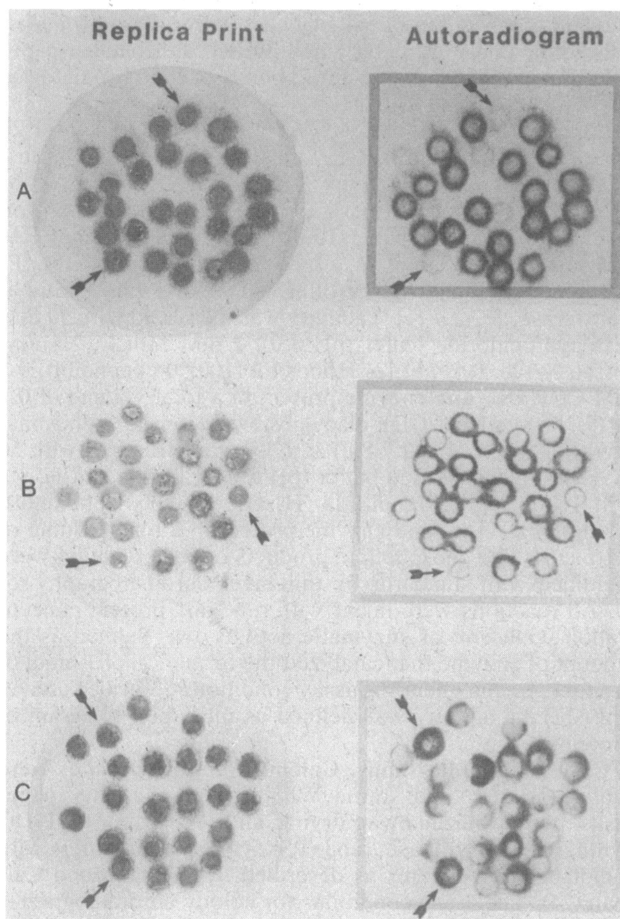

FIG. 1. Colony autoradiographic assays for phospholipid biosynthetic enzymes. A mixed culture of wild-type *ade5* and mutant *cdg1 ade5* strains was grown on YEPD plates. The colonies were replica printed onto filter paper and subjected to colony autoradiographic analysis for CDP-DG synthase (A), PI synthase (B), and PS synthase (C) activities as described in the text. The arrows reflect the enzyme activity associated with mutant *cdg1 ade5* colonies.

mined by the method of Bradford (6), using bovine serum albumin as the standard.

RESULTS

Colony autoradiographic analysis of phospholipid biosynthetic enzymes. The *cdg1* mutant was isolated by a screening method designed to detect mutants with an Opi$^-$ phenotype. The mutant was subsequently subjected to colony autoradiographic analysis of phospholipid biosynthetic enzymes to obtain preliminary information on the nature of its biochemical defect(s). A mixed culture of wild-type and mutant cells was grown on YEPD plates and replica printed to filter paper, followed by colony autoradiographic analysis of CDP-DG synthase, PI synthase, and PS synthase activities (Fig. 1). The radiolabeled phospholipids synthesized by each strain were detected as black halos on autoradiograms, reflecting the activities of each enzyme from cells present on the stained replica prints. Light halos on the autoradiogram generated by colonies identified as *cdg1* mutants showed reduced CDP-DG synthesis when compared with wild-type cells (Fig. 1A). Colonies of *cdg1* mutants synthesized less PI (Fig. 1B) and more PS (Fig. 1C) when compared with

TABLE 2. Pulse-labeling of phospholipids of wild-type and mutant strains[a]

Genotype of strain	Inositol (μM)	Phospholipid (%)						
		PA	CDP-DG	PI	PS	PE	PC	Other[b]
ade5	0	12	6	25	26	13	12	7
	50	5	2	45	18	11	13	6
opi1	0	9	2	40	19	9	16	5
	50	8	1	42	18	9	17	5
cdg1 ade5	0	15	1	26	24	9	17	8
	50	15	1	28	23	8	18	7

[a] Cells were labeled for 15 min with $^{32}P_i$ in complete synthetic medium in the absence and presence of inositol as described in the text.
[b] Includes the pooled percentages of the minor phospholipids cardiolipin, phosphatidylglycerol, PMME, PDME, and other unidentified lipids present in trace amounts.

wild-type cells. For the analysis of PS synthase activity, reaction conditions were poised so that colonies with wild-type activity had faint halos.

Phospholipid synthesis and composition of the *cdg1* mutant. Mutant and wild-type strains were pulse-labeled with $^{32}P_i$ to examine total phospholipid synthesis (Table 2). The percentages of $^{32}P_i$ incorporated into each phospholipid represent the relative rates of synthesis during the pulse. Phospholipids such as PI and PS were labeled rapidly because they are synthesized directly from CDP-DG (39). In contrast, PC was not heavily labeled in the pulse, because in the absence of exogenous choline, PC is synthesized primarily via methylation of PE (39). As previously reported for wild-type yeast (17, 30), the relative proportion of synthesized PI increased when the cells were grown in the presence of inositol. The *cdg1* strain appeared to synthesize wild-type proportions of PI when grown in the absence of inositol. However, the proportion of PI did not increase when the *cdg1* strain was grown in the presence of inositol. In addition, in the *cdg1* strain the proportion of label in PA was higher than in wild-type cells and the proportion of label in CDP-DG was lower. In contrast, the inositol-overproducing *opi1* mutant incorporated a higher proportion of label into PI whether or not it was exposed to inositol.

To analyze phospholipid composition, mutant and wild-type cells were labeled to steady state with $^{32}P_i$ (Table 3). The proportion of PI in the phospholipid composition of wild-type cells was approximately 11% when the cells were grown in the absence of inositol. However, the proportion of PI increased to 25% when wild-type cells were grown in the presence of 50 μM inositol. In contrast, the *opi1* mutant that constitutively overproduces inositol (16) had a high proportion of PI (25%) in its phospholipid composition whether or not inositol was present in the growth medium. The *cdg1* mutant did not resemble either *opi1* or wild-type strains with regard to PI composition. The level of PI in the phospholipid composition of *cdg1* strains was slightly elevated relative to the wild-type when the mutant was grown in the absence of inositol (15 versus 11%) but was not as high as in the constitutive inositol-excreting *opi1* strain (25%). In contrast to wild-type cells, the proportion of PI in the phospholipid composition of the *cdg1* mutant did not increase when cells were grown in the presence of inositol. This characteristic phospholipid composition was found in all *cdg1* strains examined, including the *cdg1 ino1* strain, which was auxotrophic for inositol.

The pattern of synthesis of methylated phospholipids was also examined in wild-type and *cdg1* strains in vivo by

TABLE 3. Phospholipid composition of wild-type and mutant strains[a]

Genotype of strain	Inositol (μM)	Phospholipid (%)						
		PA	CDP-DG	PI	PS	PE	PC	Other[b]
ade5	0	4	3	11	9	16	49	8
	50	2	2	25	7	17	40	7
opi1-1	0	3	2	25	7	17	39	7
	50	2	2	27	7	16	41	5
ino1-13	50	2	2	26	6	15	43	6
cdg1 ade5	0	5	1	15	8	14	53	4
	50	4	1	15	8	16	45	5
cdg1 ino1-13	50	5	<1	15	8	15	45	6
cdg1 leu2	50	5	<1	14	8	15	51	7
cdg1 outcross (wild type for other markers)	50	4	<1	14	9	15	52	6

[a] Cells were grown in complete synthetic medium in the absence and presence of inositol, and the steady-state phospholipid composition was determined by $^{32}P_i$ labeling as described in the text.

[b] Includes the pooled percentages of the minor phospholipids cardiolipin, phosphatidylglycerol, PMME, PDME, and other unidentified lipids present in trace amounts.

pulse-labeling with [*methyl*-^{14}C]methionine (Table 4). Wild-type cells, as previously reported (30, 35), exhibited different patterns of labeling of the methylated phospholipids PMME, PDME, and PC, depending on whether or not the cells were exposed to inositol plus choline in the growth medium. However, the *cdg1* mutant exhibited a constant pattern of synthesis of methylated phospholipids that did not change in response to the availability of the soluble precursors inositol plus choline. The proportion of label in PC was elevated in the *cdg1* strain and most resembled the proportion of PC synthesized in the wild-type strain grown in the absence of water-soluble precursors, a growth condition that corresponds to derepression of the phospholipid *N*-methyltransferases (30, 35, 41).

In vitro enzyme assays of phospholipid biosynthetic enzymes. Cell extracts were prepared from wild-type and *cdg1* cells grown in complete synthetic medium, and the activities of CDP-DG synthase, PI synthase, and PS synthase were measured. The specific activity of CDP-DG synthase in the *cdg1* mutant was 25% of the level found in wild-type cells. The specific activity of PS synthase was 50% higher in mutant *cdg1* compared to wild-type cells. In contrast to the reduced levels of PI synthase activity detected by colony autoradiography for the *cdg1* mutant, the specific activity of PI synthase for the mutant in cell extracts was similar to the activity obtained from wild-type cells. This difference is most likely due to the defect in CDP-DG synthesis in the *cdg1* mutant. This defect would result in reduced levels of endogenous CDP-DG, which was needed as substrate for the PI synthase reaction in the colony autoradiographic analysis. In the in vitro assays, CDP-DG was not limiting, since exogenous CDP-DG was added to the PI synthase reaction mixture.

Enzyme assays were performed to investigate whether the reduction in CDP-DG synthase activity and the elevation in PS synthase activity in the *cdg1* mutant were due to the presence or the loss of soluble effector molecules. Cell extracts from mutant and wild-type cells were mixed at several ratios and incubated for 20 min. Following incubation, the activities of each enzyme were measured. The specific activities of CDP-DG synthase, PS synthase, and PI synthase from the cell extract mixtures were the average of the activities of the enzymes assayed in each cell extract separately. This suggests that the decreased CDP-DG synthase activity and the increased PS synthase activity of the *cdg1* mutant were not due to the presence or absence of soluble effector molecules in one strain and not in the other. These experiments do not rule out the presence of membrane-bound effectors which could have modulated the enzyme activities.

Genetic analysis of the *cdg1* mutant. The *cdg1* mutant posed several challenges to genetic analysis. The mutant as originally isolated mated poorly, but this phenotype did not segregate with the *cdg1* lesion in subsequent crosses. However, all strains bearing the *cdg1* mutation were found to develop sterility (inability to mate) upon prolonged storage. The sterility was not further analyzed in this study, but all strains used were carefully monitored for mating ability. A further difficulty was caused by the Opi$^-$ phenotype used to monitor the *cdg1* mutation on plates. Complementation analysis and tests of dominance of the Opi$^-$ phenotype had to be performed on colony-purified diploids since haploid cells remaining in a mating mixture obscured the diploid phenotype.

The *cdg1* haploid strains generated strong inositol excretion rings in 1 day or less, whereas heterozygous diploids were found to generate a very weak excretion halo after 2 to 3 days of incubation. Tetrad analysis revealed a 2:2 segregation of the Cdg1 phenotype in 20 tetrads, confirming that a defect in a single gene was responsible for the mutant phenotype. The altered pattern of PI synthesis and the inositol excretion phenotype cosegregated in all cases with the defect in CDP-DG synthase activity.

Complementation analysis was conducted by crossing the *cdg1* mutant with representatives of the known *opi* mutant loci (i.e., *opi1*, *opi2*, *opi3*, and *opi4* [16]). The diploids resulting from these crosses all had very weak inositol excretion rings, if any. In other words, these diploids were similar in phenotype to the *cdg1* × wild-type diploid. Thus,

TABLE 4. Pulse-labeling of methylated lipids in wild-type and mutant strains[a]

Genotype of strain	Inositol (μM)	Choline (mM)	Lipid[b] (%)				
			PMME	PDME	PC	PL	NL
ade5	0	0	3	7	59	7	24
	50	1	4	9	35	10	42
cdg1 ade5	0	0	2	3	63	4	28
	50	1	1	4	63	3	29

[a] Cells were labeled for 30 min with [*methyl*-^{14}C]methionine in complete synthetic medium in the absence and presence of inositol plus choline as described in the text.

[b] PL, Polar lipids remaining near the origin; NL, neutral lipids, primarily sterols, migrating near the solvent front.

TABLE 5. Tetrad analysis of crosses of the *cdg1* mutant with other mutants

Cross	No. of asci[a]			Recombinant spores (%)
	PD	TT	NPD	
cdg1 × *ino1-13*	8	12	5	36
cdg1 × *ino2-21*	1	8	2	54
cdg1 × *ino4-39*	2	7	3	54
cdg1 × *opi1-1*	2	9	3	53
cdg1 × *opi2-37*	4	4	2	40
cdg1 × *opi3-3*	2	6	3	55
cdg1 × *opi4-4*	0	5	3	61
cdg1 × *cho2-1*	1	2	1	50

[a] PD, Parental ditype (i.e., all four spores of parental types); TT, tetratype (i.e., one spore of each parental genotype, one spore of each recombinant type); NPD, nonparental ditype (all four spores of the recombinant genotypes). Other markers segregating 2:2 include *MATa/α lys2 ade5*.

the *cdg1* mutation appears to complement mutants at all of the known *opi* mutant loci, suggesting that the *cdg1* mutation represents a new genetic locus. Tetrad analysis was performed on the crosses of *cdg1* to *opi1*, *opi2*, *opi3*, and *opi4* mutant strains. The *cdg1* mutation was found to segregate independently of the *opi1*, *opi2*, *opi3*, and *opi4* mutations (Table 5), confirming that the *cdg1* mutation represents a different genetic locus.

The *cdg1* mutant was also crossed to mutants representative of the *ino* mutant loci (*ino1-13*, *ino2-21*, and *ino4-39* [12]). Analysis of these crosses revealed spores with three phenotypes: inositol excreting, inositol requiring, and wild type (nonexcreting and nonrequiring). The existence of recombinant *ino opi* mutant spores (which, however, have an Ino$^-$ phenotype) can be inferred by the existence of the reciprocal class (INO$^+$ OPI$^+$) in a given tetrad. The *cdg1* genotype of inositol-requiring spores in selected tetrads was confirmed by genetic complementation analysis. The *cdg1* mutation was found to segregate independently of all of the *ino* mutations (Table 5). The crosses of *cdg1* to inositol-requiring strains revealed that inositol auxotrophy conferred by mutations at *ino1-13*, *ino2-21*, and *ino4-39* rendered the double-mutant haploid *cdg1 ino1-13*, *cdg1 ino2-21*, and *cdg1 ino4-39* strains inositol requiring. The double-mutant *cdg1 ino1-13* strain exhibited the phospholipid composition typical of the *cdg1* mutation alone (Table 3).

The *cdg1* mutant was also crossed to mutants *opi3* and *cho2*, which have pleiotropic defects in phospholipid metabolism. The *opi3* mutant is defective in the production of PC because of a severe reduction in the last two methylation steps leading to de novo PC production (17). However, this mutant also exhibits an Opi$^-$ phenotype, as well as altered regulation of IP synthase (17). The *cho2* mutant exhibits reduced production of PC when grown in the absence of exogenous monomethylethanolamine, dimethylethanolamine, or choline (21). The *cho2* mutant also excretes inositol under these conditions and does not regulate the *INO1* gene normally (22). Tetrad analysis (Table 5) revealed that the *cdg1* mutation readily recombines with the *cho2* and *opi3* mutations and is thus not closely linked to either. However, the *cdg1 cho2* double-mutant strain had an unusual phenotype. The *cdg1 cho2* double mutant failed to grow on complete defined medium unless it was supplemented with monomethylethanolamine or choline.

Regulation of enzymes by phospholipid precursors. CDP-DG synthase (23, 25) and PS synthase (10, 23, 30, 38) are regulated in wild-type cells by the phospholipid precursors serine, ethanolamine, and choline when grown in inositol-containing media. In the absence of inositol, these precursors have no effect on the levels of CDP-DG synthase (23, 25) and PS synthase (23, 38). Cytosolic-associated IP synthase is regulated in a similar fashion (13, 22, 30). However, the presence of inositol alone is sufficient to produce nearly maximal repression of the enzyme. The *cdg1* mutant proved to be constitutive for CDP-DG synthase and IP synthase. CDP-DG synthase activity (Table 6) and subunit (Fig. 2) levels in the *cdg1* mutant were not significantly affected by the presence of phospholipid precursors in the growth medium. The levels of the CDP-DG synthase subunit in the *cdg1* mutant were similar to those from wild-type cells when repressed by phospholipid precursors (Fig. 2). The IP synthase M_r-62,000 subunit was present at fully derepressed levels in *cdg1* cells grown in the presence of inositol alone and in combination with other phospholipid precursors (Fig. 2). Although PS synthase activity (Table 6) and subunit (data not shown) levels were regulated in the *cdg1* mutant, the magnitude of the reduction in activity in response to the precursors was about 1.3- to 2-fold less than that observed in wild-type cells (23, 38). As in wild-type cells (15, 23, 30), PI synthase activity (Table 6) and subunit (data not shown)

TABLE 6. Enzyme activities in cell extracts from wild-type and mutant strains grown in the presence of phospholipid precursors[a]

Growth medium and additions	Relative activity (%) of strains with:					
	CDP-DG synthase		PS synthase		PI synthase	
	ade5	*cdg1 ade5*	*ade5*	*cdg1 ade5*	*ade5*	*cdg1 ade5*
Complete synthetic	100	100	100	100	100	100
Complete synthetic with:						
Inositol	69	93	62	83	94	95
Serine	99	93	92	95	100	100
Inositol + serine	56	100	33	74	100	103
Ethanolamine	104	107	100	92	97	95
Inositol + ethanolamine	40	100	39	51	100	95
Choline	100	114	109	102	97	110
Inositol + choline	44	100	29	58	90	100

[a] Cells were grown in medium with 50 μM inositol, 1 mM serine, 1 mM ethanolamine, and 1 mM choline where indicated. The relative activity (%) was calculated by normalizing the specific activities (in units per milligram) of CDP-DG synthase, PS synthase, and PI synthase obtained by assay under the stated growth condition against the specific activity measured without supplementation. Specific activities were calculated from triplicate determinations from a minimum of two independent growth studies. The specific activities of CDP-DG synthase, PS synthase, and PI synthase in the *ade5* strain were 0.45, 0.41, 0.30 U/mg, respectively. The specific activities of CDP-DG synthase, PS synthase, and PI synthase in the *cdg1 ade5* strain were 0.14, 1.26, and 0.31 U/mg, respectively.

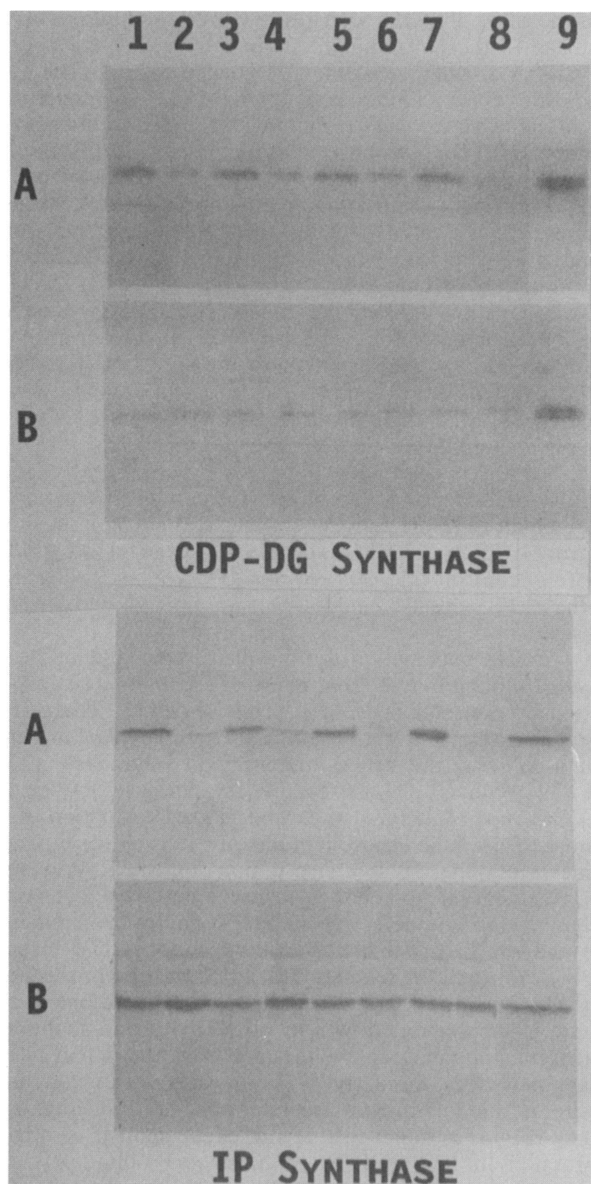

FIG. 2. Immunoblots of CDP-DG synthase and IP synthase from cell extracts of cells grown in the presence of phospholipid precursors. Shown are portions of immunoblots of the M_r-56,000 subunit of CDP-DG synthase and the M_r-62,000 subunit of IP synthase. Wild-type *ade5* (A) and mutant *cdg1 ade5* (B) strains were grown in complete synthetic medium with the following additions (lanes): no addition (1); 50 μM inositol (2); 1 mM serine (3); 50 μM inositol plus 1 mM serine (4); 1 mM ethanolamine (5); 50 μM inositol plus 1 mM ethanolamine (6); 1 mM choline (7); and 50 μM inositol plus 1 mM choline (8). Lane 9 is an immunoblot of purified CDP-DG synthase and IP synthase standards. Immunoblotting of cell extracts (75 μg) was performed as described in the text.

levels in the *cdg1* mutant were not regulated in response to water-soluble phospholipid precursors.

CDP-DG synthase activity in diploid strains. Expression of CDP-DG synthase was examined in various diploid strains. The wild-type diploid strain W303-1A/*ade5* had a derepressed level of CDP-DG synthase that was somewhat higher than that of the wild-type haploid strain *ade5* (0.91 versus 0.58 U/mg) and was regulated in response to inositol plus choline. The *cdg1* homozygous diploid strain *cdg1 leu2*/*cdg1 ade5* had a very low level of CDP-DG synthase activity (0.06 U/mg) which was slightly lower than that of *cdg1* haploid strain *cdg1 ade5* (0.11 U/mg). Heterozygous diploids *cdg1 ade5*/W303-1A and *cdg1 leu2*/*ade5* had specific activities of 0.41 and 0.72 U/mg, respectively, that were lower than the homozygous wild-type diploid activity (0.91 U/mg). Both heterozygous diploids exhibited regulated expression of CDP-DG synthase activity in response to inositol plus choline.

TABLE 7. Partial purification of CDP-DG synthase[a]

Purification step and strain genotype	Total units (nmol/min)	Protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Cell extract					
ade5	1,578	2,629	0.60	1.0	100
cdg1 ade5	381	2,541	0.15	1.0	100
Mitochondria					
ade5	1,258	505	2.49	4.1	80
cdg1 ade5	287	463	0.62	4.1	75
Triton X-100 extract					
ade5	946	228	4.15	6.9	60
cdg1 ade5	244	251	0.97	6.5	64

[a] CDP-DG synthase was partially purified from wild-type and mutant strains as described in the text. The data are calculated on the basis of 30 g (wet weight) of cells.

Partial purification and comparison of CDP-DG synthase from wild-type and mutant strains. CDP-DG synthase was partially purified from wild-type and mutant strains through the Triton X-100 solubilization step (Table 7) as described by Kelley and Carman (27). CDP-DG synthase from the *cdg1* mutant could not be purified to homogeneity because of its low level of activity. The enzyme from both strains was enriched about fourfold in the mitochondrial fraction. An additional 1.6-fold increase in specific activity was obtained by solubilization with Triton X-100. CDP-DG synthase from both strains behaved in a similar manner during purification, with proportional increases in specific activity and yield of total activity.

To investigate whether the reduction in CDP-DG synthase activity in the *cdg1* mutant was due to a distorted form of the enzyme, the basic enzymological properties of partially purified CDP-DG synthase were examined and compared with those of the wild-type enzyme. The optimum pH for the enzyme from both strains was 6.5. Maximum activity from both strains was obtained with 20 mM magnesium ions and 15 mM Triton X-100. The apparent K_m values for PA for the enzymes from the wild type and the *cdg1* mutant were 0.20 mM and 0.21 mM, respectively. The apparent K_m values for CTP for the enzymes from the wild type and the mutant were 0.11 mM and 0.12 mM, respectively. CDP-DG synthase from both strains was unstable to temperatures above 30°C, with total inactivation of activity at 60°C. The activation energy values of CDP-DG synthase for the enzyme from the wild type and the mutant were 14.5 kcal (~60.7 J)/mol and 14.9 kcal (~62.3 J)/mol, respectively. CDP-DG synthase activity from both strains behaved in a similar manner with respect to each enzymological property.

Cell extract, mitochondria, and Triton X-100-solubilized fractions of CDP-DG synthase from wild-type and mutant cells were subjected to immunoblot analysis to identify the M_r-56,000 subunit of CDP-DG synthase (Fig. 3). For each strain, the intensity of the signal for the CDP-DG synthase subunit increased as the enzyme was purified. However, the *cdg1* mutant possessed less of the CDP-DG synthase subunit in each fraction than the wild type did. The immunoblot,

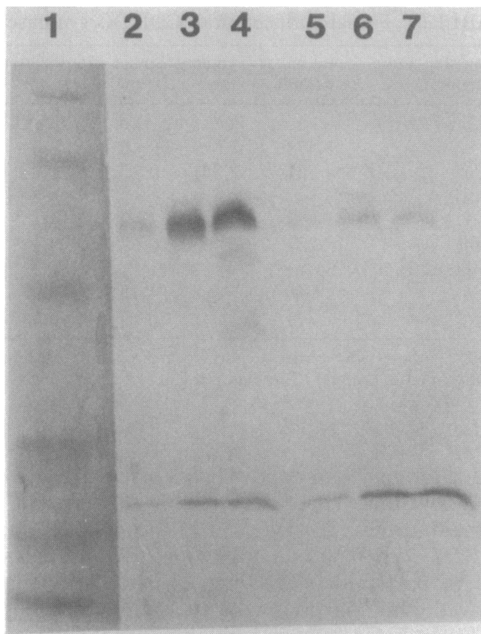

FIG. 3. Immunoblot analysis of partially purified CDP-DG synthase. Lane 1 is a Coomassie blue-stained nitrocellulose blot of protein molecular weight standards: phosphorylase *b* (M_r 92,500), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400). Lanes 2, 3, and 4 contained 75 μg each of cell extract, mitochondria, and Triton X-100 extract from the wild-type *ade5* strain. Lanes 5, 6, and 7 contained 75 μg each of cell extract, mitochondria, and Triton X-100 extract from the *cdg1 ade5* mutant. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as described in the text.

which was also analyzed for PS synthase, showed that the *cdg1* mutant had elevated levels of the PS synthase M_r-23,000 subunit compared with the wild type.

DISCUSSION

We have described the isolation and characterization of the *S. cerevisiae cdg1* mutant, which exhibited pleiotropic deficiencies in phospholipid biosynthesis. Genetic analysis revealed that the *cdg1* mutant was not allelic to any previously described genetic locus involved in phospholipid regulation. The specific activity of CDP-DG synthase in mutant haploid cells was less than 25% of the wild-type derepressed level. The results of the biochemical and immunoblot analyses revealed that the defect in CDP-DG synthase activity in the *cdg1* mutant was due to reduced CDP-DG synthase protein rather than to an alteration in the enzymological properties of the enzyme. This defect resulted in mutant cells having a reduced rate of CDP-DG synthesis and elevated PA content in pulse-labeling experiments. Furthermore, unlike wild-type cells (23, 25), CDP-DG synthase in mutant *cdg1* was not regulated in response to water-soluble phospholipid precursors.

In addition to the defect in CDP-DG synthase, the *cdg1* lesion caused constitutive overproduction of inositol and constitutive expression of IP synthase. The *cdg1* mutant also possessed elevated PS synthase activity and subunit levels. Although PS synthase in the *cdg1* mutant was regulated in response to phospholipid precursors, the magnitude of repression was less than that shown for wild-type cells (30, 38).

It is possible that the constitutive overproduction of IP synthase in the *cdg1* mutant is an attempt by the cell to provide an abundance of inositol needed to force the synthesis of PI given a limited pool of CDP-DG. PI synthase was not affected in the *cdg1* mutant. As a consequence of reduced CDP-DG synthase activity in *cdg1* cells, the PI content in the mutant was reduced compared with that in wild-type cells. On the other hand, in an attempt to also compete for a limited pool of CDP-DG, mutant cells had elevated PS synthase which was needed to synthesize PS and ultimately PE and PC.

We believe it is unlikely that the *cdg1* mutation represents a lesion in a regulatory gene involved in the control of phospholipid biosynthesis. If this were so, the *CDG1* gene would exert positive control over CDP-DG synthase and negative control over IP synthase and PS synthase. None of the phospholipid biosynthetic regulatory genes thus far described have been shown to have both negative and positive effects upon different enzymes under their control. The regulatory *INO2* and *INO4* genes exert strictly positive control (4, 13, 22, 23, 35), and the *OPI1* gene exerts strictly negative control (25, 30, 38) on IP synthase, CDP-DG synthase, PS synthase, and the phospholipid *N*-methyltransferases.

A possible explanation for the pleiotropic phenotype of the *cdg1* mutant may be that many of its characteristics are secondary consequences of a primary defect. There is a precedent for this interpretation. For example, *cho1* mutants have a defect in the structural gene for PS synthase (4, 34) and have reduced or undetectable PS synthase activity (1, 31). The *cho1* mutants also exhibit altered regulation of IP synthase (33). When these mutants are starved for ethanolamine or choline, IP synthase is not repressed by inositol (33). A similar phenomenon is observed in the *cho2* and *opi3* mutants, which are defective in the first methylation reaction (21) and the final two methylation reactions (17), respectively, leading to the formation of PC from PE. In the *cho2* mutant, IP synthase is not regulated by inositol unless the mutant is supplemented with monomethylethanolamine, dimethylethanolamine, or choline to bypass the defect in PC biosynthesis (22). Similarly, IP synthase is not regulated by inositol in the *opi3* mutant unless choline is supplied exogenously (21). It is believed that the effect upon IP synthase expression in the *cho1*, *cho2*, and *opi3* mutants is the consequence of the way in which the phospholipid regulatory network functions in *S. cerevisiae* (21). The yeast cell apparently cannot sense the presence of inositol and thus cannot regulate IP synthase and other enzymes of phospholipid biosynthesis if the reaction series leading to the synthesis of PC is interrupted (21). The primary defect in the *cho1* (4, 33, 34), *cho2* (21, 22), and *opi3* (17, 21) mutants is believed to lie in a structural gene encoding an enzyme of phospholipid biosynthesis, whereas the regulatory defects are believed to be secondary.

By analogy, it is thus possible that the *cdg1* mutant represents a lesion in a structural gene encoding a phospholipid biosynthetic enzyme. For example, the *cdg1* mutant could represent a lesion in CDP-DG synthase if this essential enzyme was encoded by more than one copy in the genome. The fact that heterozygous *cdg1* diploids had somewhat less CDP-DG synthase activity than fully repressed wild-type diploids is compatible with such a notion. However, the evidence presented here is not sufficient to permit the nature of the *cdg1* lesion to be unambiguously identified. Clearly the *CDG1* gene product must play a vital role in the synthesis or regulation or both of CDP-DG and other phospholipids.

Isolation and characterization of the *CDG1* gene will no doubt be required to clarify whether it encodes a regulatory or structural gene product.

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