Pleiotropic Alterations in Lipid Metabolism in Yeast \textit{sac1} Mutants: Relationship to “Bypass Sec14p” and Inositol Auxotrophy

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Sac1p dysfunction results in bypass of the requirement for phosphatidylinositol transfer protein (Sec14p) function in yeast Golgi processes. This effect is accompanied by alterations in inositol phospholipid metabolism and inositol auxotrophy. Elucidation of how \textit{sac1} mutants effect “bypass Sec14p” will provide insights into Sec14p function in vivo. We now report that, in addition to a dramatic accumulation of phosphatidylinositol-4-phosphate, \textit{sac1} mutants also exhibit a specific acceleration of phosphatidylcholine biosynthesis via the CDP-choline pathway. This phosphatidylcholine metabolic phenotype is sensitive to the two physiological challenges that abolish bypass Sec14p in \textit{sac1} strains; i.e. phospholipase D inactivation and expression of bacterial diacylglycerol (DAG) kinase. Moreover, we demonstrate that accumulation of phosphatidylinositol-4-phosphate in \textit{sac1} mutants is insufficient to effect bypass Sec14p. These data support a model in which phospholipase D activity contributes to generation of DAG that, in turn, effects bypass Sec14p. A significant fate for this DAG is consumption by the CDP-choline pathway. Finally, we determine that CDP-choline pathway activity contributes to the inositol auxotrophy of \textit{sac1} strains in a novel manner that does not involve obvious defects in transcriptional expression of the \textit{INO1} gene.

INTRODUCTION

The yeast Sec14p is essential for glycoprotein transport from the Golgi complex and for cell viability (Novick et al., 1980; Bankaitis et al., 1989, 1990; Franzusoff and Schekman, 1989). In the absence of Sec14p function, vesicles fail to bud from the Golgi, and secretory proteins accumulate within this organelle. The role of Sec14p in stimulating Golgi secretory function appears to be a direct one, because a pool of Sec14p exists in a specific association with yeast Golgi membranes in vivo (Cleves et al., 1991b).

Mutations in any one of at least seven genes effect an efficient bypass of the essential Sec14p requirement for Golgi function and cell viability (Cleves et al., 1991a; Alb et al., 1996; Fang et al., 1996; Kearns et al., 1998). Three of these “bypass Sec14p” loci define structural enzymes of the CDP-choline pathway for phosphatidylcholine (PtdCho) biosynthesis (Cleves et al., 1991b; McGee et al., 1994), one of the two pathways for PtdCho biosynthesis in yeast (Figure 1). Both genetic and biochemical evidence suggests that the PtdCho-bound form of Sec14p downregulates the CDP-choline pathway by effecting an inhibition of CCTase, the rate-
determining enzyme of the CDP-choline pathway (McGee et al., 1994; Skinner et al., 1995). These data exemplify how a fundamentally antagonistic relationship can exist between household phospholipid biosynthesis and essential cellular processes.

Yeast sac1 strains also exhibit bypass Sec14p phenotypes, and these strains are characterized by unscored inositol auxotrophies (Cleves et al., 1989; Whitters et al., 1993; Kearns et al., 1997). Although the molecular basis of the inositol auxotrophy of sac1 strains has not been characterized, considerable data have been obtained regarding the role of Sac1p in regulating inositol phospholipid metabolism and Golgi secretory function. Sac1p is an integral membrane protein of the Golgi and endoplasmic reticulum, and Sac1p defects result in a bypass Sec14p phenotype, an inositol auxotrophy, a cold sensitivity for growth, allele-specific suppression of yeast actin mutations, and dramatic alterations in inositol phospholipid metabolism (Cleves et al., 1989; Novick et al., 1989; Whitters et al., 1993; Kearns et al., 1997). We proposed that Sac1p dysfunction effects its bypass Sec14p phenotypes by resulting in an expansion of a Golgi diacylglycerol (DAG) pool that is required for secretory vesicle formation (Kearns et al., 1997). Because the CDP-choline pathway is a potent DAG consumer (Figure 1), we further proposed that the toxic effect of CDP-choline pathway activity on Golgi membrane secretory function is related to depletion of this Golgi DAG pool when Sec14p is absent (McGee et al., 1994; Kearns et al., 1997).

In this report, we describe the pleiotropic changes in lipid metabolism that occur in sac1 strains. Three conclusions are derived. First, sac1 yeast strains exhibit a dramatic elevation in metabolic flux through the CDP-choline pathway for PtdCho biosynthesis. Genetic and biochemical data suggest that these increased rates of PtdCho biosynthesis are driven by elevated amounts of DAG produced in sac1 strains, and that this DAG production is phospholipase D (PLD) dependent. The hyperactivity of the CDP-choline pathway (an index of increased DAG availability) correlates with all conditions under which sac1 mutations are known to support bypass Sec14p. By contrast, the massive accumulation of inositol phospholipid that is a signature of sac1 strains is by itself insufficient to effect bypass Sec14p. Second, we find that CDP-choline pathway activity in sac1 strains contributes to the Ino2 phenotype of these mutants. Finally, we demonstrate that the Ino2 phenotype of sac1 strains is not the result of inability to induce INO1 expression under conditions of inositol depletion. These results provide the first example of which we are aware regarding an inositol auxotrophy in yeast that occurs via mechanisms independent of INO1 transcriptional defects.

MATERIALS AND METHODS

Yeasts Strains

The yeast strains used in this study included: CTY182 (MATa ura3-52, 1psi-2-801, Δhis3-200), CTY1-1A (CTY182 sec14-1ts), CTY100 (CTY1-1A sac1-26), CTY243 (CTY1-1A sac1Δ-356::HIS3), CTY165 (MATa ura3-52, ade2-101, Δhis3-200, sac1-22), CTY244 (CTY182, sac1Δ-356::HIS3), CTY1079 (CTY1-1A spo14Δ::HIS3), CTY1127 (CTY100 spo14Δ::URA3), CTY1129 (CTY124 spo14Δ::HIS3), CTY1098 (CTY159 spo14Δ::URA3), and CTY1099 (CTY160 spo14Δ::URA3).

Media and Genetic Techniques

YPD and defined yeast minimal medium either containing (I+) or lacking (I-) inositol have been described (Sherman et al., 1983). Plasmids used in this study have also been described (Kagiwada et al., 1996; Kearns et al., 1997). [14C]Choline chloride, [methyl-14C]methionine, and [32P]orthophosphate were purchased from Amersham (Arlington Heights, IL). myo-Inositol and other media ingredients were purchased from Sigma (St. Louis, MO). Standard yeast genetic
methods and procedures for transformation have been described (Ito et al., 1983; Rothstein, 1983).

\[^{14}C\]Choline and \[^{14}C\]Methyl-Methionine Labeling of Yeast Lipids

Yeast strains were grown to midlogarithmic growth phase (3 ml; OD,000 = 0.8–1.0) in defined minimal medium containing 0.1 mM inositol and 1 mM choline and presented with \[^{14}C\]choline chloride (1 μCi/ml) for 20 min at 25°C with shaking (McGee et al., 1994). For \[^{14}C\]methyl-methionine labelings, strains were grown and labeled as previously described (McGee et al., 1994). Incorporation of label was terminated by the addition of trichloroacetic acid (TCA) to 5%; the cells were subsequently incubated in 5% TCA for 20 min on ice and washed once in 5% TCA, and lipids were extracted by the method of Atkinson (1984). Briefly, after washing, yeast cells were pelleted by low-speed centrifugation in a clinical centrifuge, and the pellets were resuspended in 1 ml of polar extraction solvent (McGee et al., 1994) for 20 min at 65°C. Lipids were recovered by the addition of 5 ml of CHCl₃:CH₃OH:butylated hydroxytoluene (2:1.0:0.0005%) and 0.5 ml of H₂O, followed by vigorous vortexing for 0.5 min. The mixed organic solutions were centrifuged in a clinical centrifuge for 5 min to separate the organic and aqueous phases. The organic phase was removed and dried under a gentle stream of N₂ gas, followed by resuspension of the dried lipids in 60 ml of CHCl₃:CH₃OH:butylated hydroxytoluene for resolution by one-dimensional paper chromatography using Whatman (Maidstone, United Kingdom) SG81 paper treated as described by Steiner and Lester (1972) and the solvent system CHCl₃:CH₃OH:NH₄OH (22:5.7:1). Radiolabeled PtdCho was visualized and quantitated using the PhosphorImager 425 instrument (Molecular Dynamics, Sunnyvale, CA).

For normalization of \[^{14}C\]choline incorporation into PtdCho, identical cultures were labeled with \[^{32}P\]orthophosphate (10 μCi/ml) for the same time as \[^{14}C\]choline chloride-labeled cultures. After TCA precipitation and washing, 1/10 of the culture volume was removed, immobilized on 0.5-mm glass fiber filters, and washed with 50 vol of 50 mM phosphate buffer, pH 7.0. The immobilized cells and filters were dried and placed in scintillation vials for counting. \[^{32}P\]Orthophosphate incorporation values were used to normalize loading of lipid samples from \[^{14}C\]choline-labeled cells. This was done to compensate for the fact that strains that efficiently incorporate \[^{14}C\]choline into PtdCho invariably incorporate more total \[^{14}C\]choline label into cells.

**Determination of Bulk Phospholipid Content by Radiolabeling with \[^{32}P\]Orthophosphate**

Yeast strains were grown to midlogarithmic growth phase (3 ml; OD,000 = 0.8–1.0) in defined minimal medium containing 0.1 mM inositol and 1 mM choline and presented with \[^{32}P\]orthophosphate (10 μCi/ml) for 20 min at 25°C with shaking. One-tenth of the culture was removed after TCA precipitation to assess incorporation of label. Lipids were extracted as for \[^{14}C\]choline chloride-labeled cultures, and equal amounts of incorporated counts were resolved by two-dimensional paper chromatography using Whatman SG81 paper. The first-dimension solvent was CHCl₃:CH₃OH:NH₄OH:H₂O (22:9:1:0.26), and the second-dimension solvent was CHCl₃:CH₃OH:CH₃COOH:H₂O (15:3:2:3:1). Lipids were visualized by autoradiography.

Assignment of the accumulated inositol phospholipid in sac1 mutants as PtdIns-4-Phosphate (PtdIns-4-P) was as follows. CTV182 (wild-type) and CTV244 (Sac1) cells were radiolabeled to steady state with \[^{3}H\]myo-inositol in medium supplemented with inositol (50 μM), and bulk cellular lipids were extracted and deacylated by base hydrolysis as described by Stolz et al. (1998). In parallel experiments, sac1-22 mutants were pulse radiolabeled with \[^{3}H\]myo-inositol (8 μCi/ml) for 45 min at 25°C in inositol (100 μM)-supplemented medium. An internal \[^{32}P\]PtdIns-4-P standard was generated by preparing \[^{32}P\]PtdIns-4-P from radiolabeled COS cells (York and Majerus, 1994) and including this material in the deacylation mixture with lipids recovered from the sac1-22 mutant. The deacylated products were equilibrated to 10 mM ammonium phosphate, pH 3.5, applied to a 4.6 × 125-mm Partisphere SAX-10 column (Whatman), and eluted with a linear gradient of 10–340 mM ammonium phosphate over 15 min, 340–1.02 M ammonium phosphate over 7.5 min, and isotonic 1.02 M ammonium phosphate for 5 min. Deglyceration of glycerophosphoinositol and glycerophosphoinositol phosphates was performed as described (Lips et al., 1989). Enzymatic digests of deglycerated samples were performed using recombinant inositol polyphosphate 1-phosphatase (York et al., 1994). Some 3.6 × 10⁵ cpm of deglycerated sample from sac1 mutants was incubated with mock control or with 0.4 μg of 1-phosphatase in 170 mM HEPES, pH 7.5, 1 mM EGTA, 100 mM KCl, and 3 mM MgCl₂ at 37°C for 30 min in a total volume of 50 μl. Products were analyzed by HPLC as above.

**Northern Analyses**

Total RNA was extracted from cells, resolved by electrophoresis in formaldehyde–agarose gels, and transferred to a Biodyne A membrane (Pall, East Hills, NY) as described (Hosaka et al., 1992). Specific probes for INO1, OPI3, and PPS1 were generated from appropriate gene fragments labeled with α-²²PdCTP using the Megaprime DNA random-priming system marketed by Amersham.

**Assessment of the Effects of Inositol Starvation on Cell Growth and Viability**

Yeast strains were grown to midlogarithmic growth phase in minimal defined medium containing 1 mM inositol. At time "zero" the cells were washed twice with double distilled H₂O, washed once with minimal defined medium lacking inositol (1'), and resuspended in 3 ml of the same I' medium at a cell density of 1 × 10⁶ cells/ml. At appropriate times after shift, 50 μl of culture volume were removed and serially diluted for plating onto solid complex medium (YPD plates; Sherman et al., 1983). After 3 d of growth at 25°C, colony-forming units were counted to assess viable cell numbers.

**RESULTS**

**Sac1 Yeast Strains Accumulate PtdIns-4-P**

Physiological abnormalities associated with Sac1p defects in yeast include 1) bypass Sec14p, 2) cold sensi-

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tivity for growth, 3) accumulation of an inositol phospholipid, and 4) inositol auxotrophy (Cleves et al., 1989; Kearns et al., 1997). With regard to alterations in inositol phospholipid metabolism, this accumulated inositol phospholipid is detected in lipid extracts prepared from sac1 mutants labeled with either $^{32}$P orthophosphate (Figure 2A) or $^3$H inositol (see below). This spot 1 phospholipid was originally identified by us to be the inositol sphingolipid M(IP)$_2$C on the basis of its $R_p$, our ability to label this species either with $^3$H inositol or $^{32}$P orthophosphate, and by staining with orcinol (Kearns et al., 1997). That our assignment of the spot 1 phospholipid was incorrect became clear when 1) we confirmed the finding of Stock et al. (1999) that disruption of IPT1 (the gene for M(IP)$_2$C synthase) failed to influence the bypass of the inositol phospholipid metabolism, this accumulated species that coeluted with the inositol-1,4-bisphosphate (Ins-1,4-P$_2$) standard was present at levels approximately sixfold in excess of the head group derived from PtdIns-3-P. To probe the orientation of the phosphates on the deglycerated head group, we assessed susceptibility of the soluble head group species to the action of inositol polyphosphate-1-phosphatase, an enzyme that exhibits substrate selectivity for Ins-1,4-P$_2$ (York et al., 1994). A product exhibiting the properties of Ins-4-P was generated in this enzymatic reaction (Figure 2C, lower panel), and this species was formed at the expense of the material that coeluted with the Ins-1,4-P$_2$ standard (Figure 2C, compare upper and lower panels). These data demonstrate that the spot 1 phospholipid that accumulates in sac1 mutants represents PtdIns-4-P.

Which PtdIns 4-kinase is responsible for generating the accumulated PtdIns-4-P? $^3$H inositol pulse-labeling experiments indicated that prechallenge of sac1 strains with high concentrations of wortmannin (1 mM; i.e., >1000 times the minimal growth-inhibitory concentration for sac1 mutants) had little effect on PtdIns-4-P accumulation in these strains (our unpublished results). Because wortmannin targets the Stt4p PtdIns 4-kinase, but not the Pik1p PtdIns 4-kinase, in yeast (Cutler et al., 1997), we think it likely that the PtdIns-4-P that accumulates in sac1 strains is primarily generated via Pik1p.

**Sac1 Yeast Strains Experience Elevated Flux through the CDP-Choline Pathway for PtdCho Biosynthesis**

Examination of the data presented in Figure 2A revealed several additional abnormalities in sac1 strains in addition to accumulation of PtdIns-4-P. First, incorporation of $^{32}$P radiolabel into PtdCho appeared to be greatly enhanced in sac1-22 strains relative to its SAC1 partner. Second, sac1-22 strains exhibited reduced levels of phosphatidylycerine (PtdSer). The sac1-22 allele is unique in that it is the only sac1 mutation that does not evoke inositol auxotrophy. Nevertheless, the bypass Sec14p phenotype associated with this allele, and the other manifest alterations in inositol phospholipid metabolism associated with it are inositol dependent (Kearns et al., 1997).

To more closely examine the relationship between Sac1p function and phospholipid metabolism, we quantitated the rates of phospholipid biosynthesis in sac1-22, sac1-26, and Δsac1 strains and a SAC1 partner strain. Yeast were pulse radiolabeled in inositol-containing medium for 20 min with $^{32}$P orthophosphate at 25°C, phospholipids were extracted by HPLC. As shown in Figure 2C (upper panel), the expected head group species were recovered as judged by their coelution with appropriate inositol phosphate standards. Consistent with the analyses of decylated inositol glycerolipids from sac1 strains, the accumulated species that coeluted with the inositol-1,4-bisphosphate (Ins-1,4-P$_2$) standard was present at levels approximately sixfold in excess of the head group derived from PtdIns-3-P. To probe the orientation of the phosphates on the deglycerated head group, we assessed susceptibility of the soluble head group species to the action of inositol polyphosphate-1-phosphatase, an enzyme that exhibits substrate selectivity for Ins-1,4-P$_2$ (York et al., 1994). A product exhibiting the properties of Ins-4-P was generated in this enzymatic reaction (Figure 2C, lower panel), and this species was formed at the expense of the material that coeluted with the Ins-1,4-P$_2$ standard (Figure 2C, compare upper and lower panels). These data demonstrate that the spot 1 phospholipid that accumulates in sac1 mutants represents PtdIns-4-P.
methods optimal for recovery of the major yeast phospholipids, and individual phospholipid species were resolved by paper chromatography and quantitated (see MATERIALS AND METHODS). The results are shown in Figure 3A. The data demonstrate that sac1 mutants indeed exhibited dramatically increased rates of PtdCho biosynthesis. Quantitation of the phospholipid profiles revealed that the sac1-22, sac1-26 and Δsac1 mutants all incorporated nearly 70% more [32P]orthophosphate into PtdCho during the 20-min pulse than did the SAC1 strain (Figure 3A). This difference could not be attributed to generally altered rates of phospholipid synthesis in sac1 strains, because sac1 and wild-type strains exhibited very similar rates of incorporation of 32P into phospholipid (see legend to Figure 3A). Because SAC1 and sac1 strains incorporated very similar amounts of [32P] into phospholipid per OD600 cells during the labeling period, these data indicate that sac1 mutants produced some 70% more PtdCho on a per cell basis than did wild-type strains during the labeling period (Figure 3A). Elevated PtdCho production was discerned only in pulse-radio-labeling experiments, however. No significant difference in the steady-state PtdCho content of SAC1 and mutant sac1 strains was recorded (our unpublished results). Decreased amounts of radiolabel in the PtdIns fraction were also recovered from these sac1 mutant strains. Finally, these pleiotropic sac1-associated effects on phospholipid metabolism were recorded irre-

Figure 2. Phospholipid profiles of wild-type and sac1 mutant yeast strains. (A) Yeast strains were pulse radiolabeled for 20 min with [32P]orthophosphate at 26°C in inositol- and choline-supplemented medium. Radiolabeled phospholipids were extracted using an acidic extraction solvent suitable for recovery of phosphoinositides (see MATERIALS AND METHODS) and resolved by two-dimensional paper chromatography. Phospholipid species are numbered.

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Figure 3. Phosphatidylcholine synthesis in wild-type and sac1 mutant yeast strains. (A) Strains with the indicated genotypes (at bottom) were grown to midlogarithmic growth phase in medium containing inositol (0.1 mM) and choline (1 mM). Cell cultures were then pulse radiolabeled with $^{32}$Porthophosphate (10 $\mu$Ci/ml) for 30 min at 25°C. Phospholipids were extracted by the method of Atkinson (1984) and resolved by two-dimensional chromatography, and radiolabeled species were quantified by phosphorimaging (see MATERIALS AND METHODS). Biosynthetic rates were deduced from the relative proportion of label recovered in each individual phospholipid species (expressed as percentage of total phospholipid). Rates of total phospholipid synthesis in these strains were estimated by measuring incorporation of $^{32}$P into chloroform-soluble counts (11,300–12,600 cpm/OD$_{600}$ for sac1 and sac1, $\Delta$spo14 mutants to 10,000–11,000 cpm/OD$_{600}$ for wild-type strains). Error bars are indicated. Phospholipid species are presented in the following order: PtdIns (black bars), PtdCho (cross-hatched bars), PtdSer (hatched bars), PtdEtn (stippled bars), and phosphatidic acid (open bars). Strains used were CTY1-1A (sec14-1ts, SAC1, SPO14), CTY165 (sec14-1ts, sac1-22, SPO14), and CTY1261 (sec14-1ts, sac1-22, $\Delta$spo14). These data represent an average of three independent experiments, and all strains were analyzed in parallel for each trial. In a typical quantitation, PtdCho accounted for $5.0 \times 10^5$ and $2.7 \times 10^5$ phosphorimager units in sac1 and SAC1 strains, respectively, when $1 \times 10^6$ phosphorimager units of total phospholipid were analyzed from each strain. (B) Activities of the CDP-choline pathway and PtdEtn methylation pathway were evaluated in vivo by pulse radiolabeling of SAC1 and sac1-22 strain pairs (CTY1-1A and CTY165, respectively). The indicated strains were grown to midlogarithmic growth phase in choline-free medium containing inositol. Cell cultures were split, and one aliquot was pulse radiolabeled with $^{[14}C$]choline chloride ($1 \mu$Ci/ml) or $^{[14}C$]methyl-methionine ($1 \mu$Ci/ml), respectively, for 20 min at 26°C with shaking. A parallel aliquot was labeled with $^{32}$Porthophosphate for 20 min at 26°C. Phospholipids from equal cell equivalents (determined by $^{32}$P incorporation into phospholipid in the parallel cultures) were subsequently extracted, resolved, and quantified as described in MATERIALS AND METHODS. These data represent an average of three independent experiments. For quantitative comparison of a representative experiment, wild-type and sac1 strains incorporated $3.8 \times 10^4$ and $1.3 \times 10^5$ cpm $^{[14}C]$choline chloride into PtdCho per OD$_{600}$ cells, respectively. (C) Strains with the indicated
spective of whether these strains harbored SEC14 or sec14<sup>−</sup> alleles. At this point, we do not know what contribution reduced PtdSer levels make to the various sac1 phenotypes.

Yeast produce PtdCho either via the CDP-choline pathway or the phosphatidylethanolamine (PtdEtn) methylation pathway (Figure 1). We determined whether the increased PtdCho biosynthetic rates observed in sac1 mutants resulted from increased activity of one or both of these pathways. To specifically monitor activity of the CDP-choline pathway, we subjected sac1-22 and SAC1 strain pairs to a [methyl-<sup>14</sup>C]choline pulse-radiolabeling regimen and measured the incorporation of radiolabel into PtdCho (see MATERIALS AND METHODS). The data indicate that sac1-22 mutants incorporated 2.8 ± 0.6-fold more [methyl-<sup>14</sup>C]choline into PtdCho per OD<sub>600</sub> of cells than did SAC1 strains, and this effect was typical for sac1 mutants (Figure 3B).

We interpret these data to indicate that the sac1 mutants sustain a rate of metabolic flux through the CDP-choline pathway that is two- to threefold greater than that exhibited by wild-type yeast.

Two independent lines of evidence further support this finding. First, [<sup>32</sup>P]orthophosphate pulse-radiolabeling experiments demonstrated that omission of choline from the medium (i.e., a condition that reduces CDP-choline pathway activity) diminished the rate of <sup>32</sup>P radiolabel incorporation into PtdCho. Under those conditions, sac1 mutants exhibited only a 1.3-fold greater rate of <sup>32</sup>P incorporation into PtdCho relative to wild type. Second, genetic disruption of the CDP-choline pathway in Δsac1 strains reduced flux through the CDP-choline pathway some 30-fold (as measured by [methyl-<sup>14</sup>C]choline incorporation into PtdCho in a 20-min pulse). The resultant basal level of CDP-choline pathway activity was similar to that measured for an isogenic SAC1,cki1-284::HIS3 strain (our unpublished results). These findings excluded the formal possibility that a normally cryptic choline- and DAG-utilizing pathway for PtdCho synthesis was activated in sac1 mutants.

To determine whether the PtdEtn methylation pathway for PtdCho biosynthesis was also stimulated in sac1 mutants, a [methyl-<sup>14</sup>C]methionine pulse-radiolabeling strategy was used to measure the activity of this pathway (see Figure 1). No significant differences in [methyl-<sup>14</sup>C]methionine incorporation into PtdCho were recorded between sac1 and SAC1 strains, because the sac1 mutant exhibited 1.2 ± 0.4 the wild-type rate of PtdEtn methylation pathway activity (Figure 3B).

**DAG Kinase (DGK) Expression Reduces CDP-Choline Pathway Activity in sac1 Strains**

The CDP-choline pathway directly consumes DAG in the process of PtdCho synthesis, whereas the methylation pathway does not (Figure 1). Pulse-radiolabeling analyses described above demonstrated that sac1 strains produce DAG at rates some two- to threefold greater than those recorded for isogenic SAC1 strains, suggesting that CDP-choline pathway hyperactivity in sac1 strains might be supported by increased DAG availability. To investigate the issue in more detail, *Escherichia coli* DGK, an enzyme that converts DAG to phosphatidic acid (PtdOH), was expressed in Δsac1 strains. We then assessed the effect of metabolic shunting of DAG to PtdOH on CDP-choline pathway activity in Δsac1 strains.

[<sup>14</sup>C]Choline radiolabeling experiments demonstrated that DGK expression effected a reproducible 30% decrease in CDP-choline pathway activity in sac1 strains (Figure 3C). Although the effect was relatively modest, and the rate of CDP-choline pathway activity in DGK-expressing Δsac1 strains still exceeded those of wild-type strains, this reduction in CDP-choline pathway was physiologically significant, because it strongly influenced sac1-associated inositol auxotrophy (see below).

**Elevated CDP-Choline Pathway Activity in Δsac1 Strains Is PLD Dependent**

Full manifestation of all presently known bypass Sec14p mechanisms, including those associated with sac1 mutations, requires the contribution of a functional SPO14 gene, which encodes the sole PIP<sub>γ</sub>γ-activated PLD in yeast (Xie et al., 1998). In addition, the bypass Sec14p phenotype of sac1 strains is abolished by DGK expression (Kearns et al., 1997). We have proposed that PLD may play a role in DAG generation by providing a pool of PtdOH that serves as a substrate for PtdOH phosphohydrolases. A prediction of this model is that PLD is required to sustain the elevated CDP-choline pathway activity of sac1 strains because it ultimately generates excess DAG in these mutants.

To test this prediction, a [<sup>32</sup>P]orthophosphate pulse-radiolabeling regimen (which predominantly monitors PtdCho synthesis via the CDP-choline pathway; McGee et al., 1994) was used. In these experiments, excess choline (1 mM) was included in the growth

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**Figure 3 (cont.).** Genotypes were grown to midlogarithmic growth phase in choline-free medium containing inositol. Cell cultures were split, and one aliquot was pulse radiolabeled with [methyl-<sup>14</sup>C]choline chloride (1 μCi/ml) or [<sup>32</sup>P]orthophosphate (10 μCi/ml) for 20 min at 26°C. Phospholipids were extracted, resolved, and quantified (see MATERIALS AND METHODS). Again, samples were normalized for cell equivalents based on incorporation of [<sup>32</sup>P]orthophosphate into the parallel cultures as described in B. These data represent an average of three independent experiments. Strains CTY182 (SAC1) and CTY244 (Δsac1) were transformed with the test YEp(DGK) plasmid and the vector-only control YEp(URA3) plasmid, respectively. The YEp(DGK) plasmid was represented by the pCTY85 construct that drives constitutive expression of DGK in yeast (Kearns et al., 1997).
medium to ensure that any influence of PLD on CDP-choline pathway activity was independent of PLD-mediated generation of choline itself. Introduction of a Δspo14 allele into the sac1-26 strain evoked a significant reduction in CDP-choline pathway activity (Figure 3A). In these experiments, PtdCho represented 29.5 ± 1.9 and 49.0 ± 1.9% of the radiolabeled phospholipid extracted from SAC1 and sac1 strains, respectively. The rate of PtdCho synthesis in the sac1-26, Δspo14 mutant was not significantly different from that of wild type, because PtdCho comprised 34.5 ± 3.4% of the radiolabeled phospholipid recovered from that double mutant strain (Figure 3A). Because all three strains incorporated very similar amounts of 32P into phospholipid per OD600 cells during the labeling period (see Figure 3A legend), the relative percentages of label incorporated into PtdCho directly reflected quantitative differences in rates of PtdCho synthesis. The demonstration that sac1-26, Δspo14 double mutant strains exhibit wild-type rates of flux through the CDP-choline pathway, when coupled with the sensitivity of sac1-mediated CDP-choline pathway hyperactivity to DGK expression (Figure 3C), suggests that PLD activity supplies the DAG that drives accelerated metabolic flux through this pathway.

To determine whether PLD activity was solely responsible for the increased DAG production observed in sac1 mutants (Kearns et al., 1997), we subjected isogenic PLD-proficient (SPO14) and deficient (Δspo14) sac1-22 strain pairs to a 20-min [14C]acetate pulse at 25°C. Lipids were extracted, and DAG was resolved by TLC (Buttke and Pyle, 1982). We took care to expeditiously extract and resolve the DAG to limit the unavoidable isomerization of 1,2-DAG to 1,3-DAG. Isogenic SAC1, SPO14 strains were used as wild-type controls, and both 1,2-DAG and 1,3-DAG species were identified and quantitated to yield total DAG values. In accord with the data of Kearns et al. (1997), sac1, SPO14 strains exhibited a 70% increase in DAG production relative to wild-type control (22 ± 2 and 13 ± 1% of total extractable lipid, respectively; n = 3). This effect was observed in media with inositol concentrations of ≥300 μM, as used by Kearns et al. (1997), but not in media with inositol concentration of <100 μM. Rates of [14C]acetate incorporation into lipid in these experiments were equivalent for SAC1 strains and sac1 mutants (420 ± 60 and 418 ± 18 cpm · OD600−1 · min−1, respectively). Genetic inactivation of PLD had no effect on the elevated DAG production scored for sac1-22 strains as assayed by this regimen (21 ± 1%; n = 3).

We also used steady-state [14C]acetate labeling experiments to assess DAG pools at equilibrium in SAC1 strains, sac1-22 mutants, and sac1-22, Δspo14 double mutants. Because sac1 mutants exhibit elevated activity of at least one DAG consuming pathway (i.e., the CDP-choline pathway), we expected that sac1 mutants
would not exhibit elevated DAG levels at steady state. As predicted, no steady-state differences in DAG pools were detected in these strains (13 ± 1, 12 ± 1, and 12 ± 2% of total chloroform-soluble counts, respectively; n = 3). These data are in accord with our previous determinations in which we measured DAG by radiolabeling cells to steady state with [1-14C]acetate and chasing for 2 h. In those experiments, there also were no significant differences in steady-state DAG pools among SAC1 strains, sac1 mutants, and sac1, Δspo14 double mutants (Xie et al., 1998). These cumulative data suggest that sac1 strains produce elevated DAG by at least two distinct pathways: one that is PLD dependent and results in a DAG pool that is robustly scavenged by CDP-choline pathway activity, and a second pathway that is revealed in [14C]acetate pulse-radiolabeling experiments, when cells are grown in media containing higher concentrations of inositol.

**Accumulation of PtdIns-4-P in PLD-deficient sac1 Strains**

The bypass Sec14p phenotype of sac1 strains correlates closely with the dramatic accumulation of an inositol phospholipid (Kearns et al., 1997), which is now identified as PtdIns-4-P (Figure 2A). Perhaps elevated PtdIns-4-P defines the biochemical basis for bypass Sec14p in sac1 strains. Yet, the sac1 bypass Sec14p phenotype is sensitive to both DGK expression and genetic inactivation of PLD (Kearns et al., 1997; Xie et al., 1998). We therefore tested whether either of the conditions that abrogate sac1-mediated bypass Sec14p also influenced PtdIns-4-P accumulation in sac1 strains.

Both [3H]inositol- and [32P]orthophosphate-labeling regimens were used to compare the magnitude of PtdIns-4-P accumulation in SAC1 and sac1-22 strain pairs that were either proficient or deficient in PLD activity. As shown in Figure 4A, [3H]inositol pulse-radiolabeling experiments indicated that sac1-22 strains exhibited a dramatic (more than sixfold) accumulation of PtdIns-4-P relative to SAC1 strains when cultured in inositol-containing medium. Inspection of profile obtained for the isogenic Δspo14 genetic background, neither sac1, kes1 nor BSD1-124 alleles imparted any detectable phenotypic suppression of sec14 growth defects. By contrast, inactivation of the CDP-choline pathway via the pet1-2 mutation (and ckl1; our unpublished results) retained a clear PLD-independent ability to suppress sec14-growth defects under these conditions.

**Figure 4 (facing page). Effects of PLD deficiency on PtdIns-4-P accumulation and bypass Sec14p.** (A) Strains CTY1-1A (SAC1, SP014), CTY165 (sac1-22, SPO14), and CTY (sac1-22, Δspo14) were grown in medium containing inositol (0.1 mM) and pulse radiolabeled with [3H]inositol (5 μCi/ml) for 1 h at 25°C and shaking. Radiolabeled inositol phospholipids were subsequently extracted and resolved by two-dimensional paper chromatography. Radiolabeled species were visualized by autoradiography. The positions of PtdIns and PtdIns-4-P are identified. These data are from a representative experiment. (B) The conditions of the experiment were exactly as in A with the exception that cells were radiolabeled with [35P]orthophosphate (10 μCi/ml). Radiolabeled species were subsequently extracted under acidic conditions optimal for recovery of phosphoinositides, resolved by two-dimensional chromatography, visualized, and quantitated by phosphorimaging. The data represent the average of three independent experiments. The bulk incorporation of label into phospholipid for these strains during the pulse was essentially equivalent (see Figure 3A), and the relative magnitudes of PtdIns-4-P were directly related to quantities of PtdIns-4-P per cell equivalent in these strains. (C) A set of isogenic sec14-1-ts, Δspo14 strains carrying the indicated bypass Sec14p mutations were streaked for single colonies on YPD medium and incubated for 72 h at 33.5°C. The corresponding sac1-26, pet1-2, kes1-1, and BSD1-124 derivatives were represented by strains CTY1127, CTY1096, CTY1098, and CTY1129, respectively. The SEC14, Δspo14 derivative (CTY1092) served as a positive growth control, whereas the sec14-1-ts, Δspo14 strain (CTY1079) represented the negative control. In the Δspo14 genetic background, neither sac1, kes1 nor BSD1-124 alleles imparted any detectable phenotypic suppression of sec14 growth defects. By contrast, inactivation of the CDP-choline pathway via the pet1-2 mutation (and ckl1; our unpublished results) retained a clear PLD-independent ability to suppress sec14-growth defects under these conditions.
and the \( \Delta \text{sac1-22, } \Delta \text{spo14} \) double mutant strain was recovered in PtdIns-4-P, respectively (Figure 4B). DGK expression, which is similar to \( \Delta \text{spo14} \) in that it too abrogates sac1-mediated bypass Sec14p (Kearns et al., 1997), also had no effect on PtdIns-4-P accumulation, as measured by \([32P]\)orthophosphate pulse radiolabeling (our unpublished results). Although \( \text{sac1-22} \) strains were used in these experiments, the same results were obtained with \( \text{sac1} \) null strains as well (our unpublished results). We conclude that PtdIns-4-P accumulation is insufficient to support bypass Sec14p.

**sac1 Mutants Are Entirely Dependent on PLD Activity for Growth under Sec14p-deficient Conditions, Whereas \( \text{pct1} \) Mutants Are Not**

PLD is an essential component of the mechanism(s) by which all known classes of bypass Sec14p alleles exert their suppressor phenotypes (Sreenivas et al., 1998; Xie et al., 1998). What remains unclear is whether PLD is the ultimate executor of these bypass Sec14p phenotypes or whether it is only one of several contributory factors. To distinguish between these possibilities, PLD-deficient sec14-1ts strains carrying individual bypass Sec14p mutations were analyzed for their abilities to grow under various conditions. Although a \( \text{SEC14, } \Delta \text{spo14} \) double mutant strain grew well at 33.5°C, the isogenic sec14-1ts derivative was unable to grow at this temperature (Figure 4C). Indeed, the sec14-1ts, \( \Delta \text{spo14} \) double mutant was incapable of growth at temperatures above 31.5°C. Individual \( \text{sac1}, \text{kes1}, \) or BSD1-124 mutations failed to suppress sec14-1ts growth defects at 33.5°C (Figure 4C) or any other restrictive temperature. Thus, the mechanisms for bypass Sec14p in these strains operated through PLD because no PLD-independent component for suppression could be discerned.

Different results were obtained for sec14-1ts, \( \Delta \text{spo14} \) mutants carrying mutations (\( \text{pct1} \)) that inactivate the CDP-choline pathway. These triple mutant strains grew well at 33.5°C, clearly indicating a PLD-independent component to suppression of sec14 defects by this mechanism (Figure 4C). Analysis of sec14-1ts, \( \Delta \text{spo14} \), \( \text{ckil} \) triple mutants yielded the same results (our unpublished results). We conclude that PLD is a major (but not the sole) contributor to the bypass Sec14p condition elicited by CDP-choline pathway defects. Interestingly, steady-state \([32P]\)orthophosphate radiolabeling experiments conducted at 26°C, followed by a 2-h chase at 33.5°C, revealed that sec14-1ts, \( \Delta \text{spo14}, \text{pct1} \) strains exhibited similarly reduced bulk PtdOH levels to sec14-1ts, \( \Delta \text{spo14}, \text{sac1} \) strains. These reductions in bulk PtdOH...
were 10-fold relative to isogenic SPO14 strains (2.1 ± 0.4 vs. 0.2 ± 0.0% of total phospholipid for SPO14 and Δspo14 derivatives of the pct1 strain, respectively; n = 3). Yet the pct1 strains grew well at 33.5°C, whereas the sac1 partners did not. Thus, reduced PtdOH levels can be uncoupled from the mechanisms by which CDP-choline pathway mutations effect bypass Sec14p.

**Constitutive Transcription of INO1 Fails to Relieve sac1 Inositol Auxotrophy**

Sac1p is a multifunctional protein that regulates ATP import into the lumen of intracellular organelles in a manner that can be uncoupled from its role in regulating inositol phospholipid metabolism (Mayinger et al., 1996; Kearns et al., 1997; Köchendorfer et al., 1999). Because Δsac1 mutations evoke an unfolded protein response in yeast (Köchendorfer et al., 1999), and this response inhibits transcription of INO1, we analyzed whether the Ino⁻ phenotype of sac1 strains was related to defects in INO1 expression. The INO1 gene product catalyzes the conversion of glucose-6-phosphate to inositol-1-phosphate, the committed step for inositol biosynthesis in yeast (Carman and Henry, 1989). INO1 transcription is also strongly repressed by inositol and choline, and efficient induction of INO1 expression upon shift of yeast from inositol-replete to inositol-deficient conditions is required for cellular viability in inositol-free growth media (Culbertson et al., 1976a,b).

To address whether defects in INO1 expression contributed to the Ino⁻ phenotype of sac1 mutants, we used two independent approaches. First, the INO1 gene was placed under constitutive transcriptional control of the yeast SEC14 promoter. This PSEC14:INO1 expression cassette was introduced into ino1-13 and Δsac1 strains in the context of a high-copy yeast plasmid YEp(PSEC14:INO1). Introduction of the YEp(PSEC14:INO1) plasmid restored inositol prototrophy to ino1-13 strains, thereby providing a demonstration that YEp(PSEC14:INO1) sustained sufficient expression of INO1 to correct the inositol auxotrophy of an ino1 mutant. Strikingly, YEp(PSEC14:INO1) failed to complement the Ino⁻ phenotype of Δsac1 strains (Figure 5A).

Second, we used Northern analyses to assess the efficiency of induction of INO1 transcription upon shift of SAC1 and Δsac1 yeast strains from inositol-containing medium to inositol-free medium. INO1 mRNA was essentially undetectable in either wild-type or Δsac1 cells shifted to YPD medium to minimal medium supplemented with inositol and choline (Figure 5B). Yet, both SAC1 and Δsac1 strains induced robust transcription of INO1 by 4 h after shift from YPD to inositol- and choline-free minimal medium. Similarly, OPI3 transcription, which is regulated in the same manner as is that of INO1 (Carman and Henry, 1989), was also induced to high levels upon shift of Δsac1 strains from YPD to inositol-free minimal medium (Figure 5B). These data demonstrated that the Ino⁻ phenotype of sac1 strains is not a simple consequence of defects in INO1 expression.

**DGK Expression and CDP-Choline Pathway Defects Suppress Inositol Auxotrophy**

We noted that DGK expression strongly influenced the inositol requirement of sac1 strains. Normally, the Ino⁻ trait of sac1 strains is a tight phenotype because these mutants fail to produce colonies when streaked for isolation on inositol-free media (Figure 6A), even when such cultures are streaked with a heavy inoculum and incubated for many days. Expression of bacterial DGK in these strains suppressed the inositol auxotrophy of even Δsac1 mutants to the extent that individual colonies could readily be observed within 72 h of incubation of cells on inositol-free agar plates (Figure 6A). This effect was specific in the sense that DGK expression influences only one other sac1 phenotype; i.e., it abolishes sac1-mediated bypass Sec14p (Kearns et al., 1997). DGK expression did not diminish the cold sensitivity for growth (cs) phenotype characteristic of sac1 strains (our unpublished results).

The DGK expression data suggested the possibility that the Ino⁻ phenotype of sac1 mutants may result from accelerated CDP-choline pathway activity. A prediction of this model was that genetic inactivation of this specific PtdCho biosynthetic pathway will also restore an Ino⁻ phenotype to Δsac1 strains. To test this prediction, mutations that disrupt structural genes encoding CDP-choline pathway enzymes were introduced into Δsac1 mutants, and the abilities of the resultant double mutants to grow on inositol-free media were assessed. The data show that introduction of the cki1-284::HIS3 mutation (a disruption of the structural gene encoding choline kinase, the first enzyme of the CDP-choline pathway; see Figure 1), fully restored the ability of Δsac1 mutants to grow in inositol-free medium (Figure 6B). Phenotypic suppression of the sac1-associated inositol auxotrophy by the cki1-284::HIS3 allele was recapitulated when pct1::URA3 disruption alleles (which inactivate the structural gene encoding the second enzyme of the CDP-choline pathway; Figure 1) were introduced into Δsac1 strains (our unpublished results). Significantly, an opi3::URA3 allele (which inactivates the PtdEtn pathway for PtdCho biosynthesis; Figure 1) failed to suppress the Δsac1-associated Ino⁻ phenotype (Figure 6B). The potency with which CDP-choline pathway defects suppress the Δsac1-associated Ino⁻ phenotype was evident in shift experiments in which Δsac1, cki1-284::HIS3 double mutants displayed wild-type growth rates upon shift from inositol-replete
to inositol-free liquid media (Figure 7). In the course of these experiments, we observed a distinction between the behavior of sac1 and ino1 mutants under conditions of inositol deprivation. Both ino1-13 and Δsac1 strains retained viability up to 4 h after shift to inositol-free medium. After that time, however, the ino1-13 strain experienced a rapid loss of cell viability that resulted in a 1000-fold decrease in viable cell number by 24 h after shift (Figure 7). This is characteristic of yeast mutants unable to synthesize inositol de novo (Culbertson et al., 1976a, b; Carman and Henry, 1989). By comparison, Δsac1 mutants suffered a more modest 10-fold reduction in viable cell number after 24 h of inositol starvation.

**DISCUSSION**

A dissection of Sec14p function in yeast has been driven by the analysis of suppressor mutations that endow cells with the ability to execute Golgi function, and retain viability, in the absence of Sec14p (Cleves et al., 1991a; Kearns et al., 1998). The logic on which suppressor genetics is founded dictates that such bypass Sec14p mutations exert their effects by restoring a biochemical condition that normally falls under the purview of Sec14p function. From these analyses, we have proposed that Sec14p functions to maintain a Golgi DAG pool that is critical for Golgi secretory function (McGee et al., 1994; Kearns et al., 1997, 1998). Specifically, we proposed that PtdCho-bound Sec14p down-regulates DAG consumption via the CDP-choline pathway (Skinner et al., 1995; Alb et al., 1996; Kearns et al., 1998), whereas PtdIns-bound Sec14p promotes DAG generation by regulating inositol phospholipid metabolism (Fang et al., 1998; Kearns et al., 1997, 1998). In this manner, Sec14p serves as a phospholipid sensor whose phospholipid-bound states independently, but convergently, function to maintain Golgi DAG (Figure 8).
Figure 7. Effects of inositol starvation on cell viability. Wild-type (CTY182; closed circles), Δsac1 (CTY244; closed squares), Δsac1, Δcki1 (CTY; stippled circles), and ino1-13 (CTY; open squares) strains were grown in minimal defined medium supplemented with inositol, harvested, washed, and shifted to inositol-free medium at time zero. Samples of each culture were subsequently removed at the indicated times after shift and serially diluted in YPD medium, and the serial dilutions were immediately plated onto YPD agar plates. Individual colonies were scored after 3 d of growth at 26°C to assess viable cell counts. Data are from a representative experiment.

One of several important lines of evidence supporting the DAG model was our observation that the ability of sac1 strains to effect bypass Sec14p correlated with what we interpreted as overproduction of an inositol phospholipid that we identified as the most highly modified yeast sphingolipid, M(IP)2C, a lipid whose synthesis produces Golgi DAG (Kearns et al., 1997). In those experiments, the failure of Kearns et al. (1997) to use deacylation as an initial means for fractionating the deacylatable glycerophospholipids from sphingolipids contributed significantly to the misidentification. More rigorous analyses now indicate that the major inositol phospholipid that accumulates in sac1 strains is PtdIns-4-P (Figure 2). A biochemical basis for elevations in PtdIns-4-P is suggested by the demonstration that the Sac1p domains of other inositol phosphatase modules (Guo et al., 1999). Thus, sac1 strains likely accumulate PtdIns-4-P because the primary mechanism for its degradation to PtdIns is inactivated.

Based on the collective data reported herein, we revise our interpretation of the mechanism for bypass Sec14p in sac1 strains to take into account the various new data (Figure 8). We maintain that increased DAG production represents the key physiological event that allows Sec14p-independent growth and secretion in these strains, as previously proposed (Kearns et al., 1997). The evidence now suggests that the pathway for this DAG production in sac1 mutants involves PLD activity. We also report the unanticipated discovery that the physiological basis for sac1-associated inositol auxotrophy is related to aberrant lipid metabolism in these strains and not to defects in transcriptional induction of INO1. The evidence that speaks to these various points is as follows.

First, we demonstrate that a biochemical signature of sac1 mutants is a dramatic acceleration in the rate of metabolic flux through the CDP-choline pathway for PtdCho biosynthesis (Figure 3). This effect is observed for all sac1 alleles, including Δsac1, and it is observed in sac1-22 strains only when these strains are grown in the presence of inositol. The significance of the latter point is that sac1-22 strains, although exceptional from the standpoint that these are not inositol auxotrophs, are only able to exhibit bypass Sec14p phenotypes when grown in inositol-containing medium (Kearns et al., 1997). The signature alterations in inositol phospholipid metabolism characteristic of sac1 strains are also recorded in sac1-22 strains but, again, only when these mutants are provided with inositol in the growth medium (Kearns et al., 1997; see above).

Second, our demonstration that accelerated rates of CDP-choline pathway activity correlate with bypass Sec14p in sac1 mutants is consistent with the DAG production model shown in Figure 8. Because sac1 strains exhibited wild-type levels of bulk DAG at steady state, an expected consequence of excess DAG production in sac1 strains would be a compensatory increase in the activity of a DAG degrading–consuming pathway. We conclude that the CDP-choline pathway represents a major metabolic sink for excess DAG in sac1 strains. Our finding that CDP-choline pathway hyperactivity in sac1 strains is sensitive to the metabolic conversion of DAG to PtdOH effected by DGK expression also supports this concept (Figure 3C). That this excess DAG is ultimately produced from PtdOH, a phospholipid that is a direct product of PLD action, is suggested by the demonstration that PLD inactivation (even when exogenous choline is supplied in vast excess) reduces CDP-choline pathway activity in sac1 strains to essentially wild-type levels (Figure 3A).

The stimulation of the CDP-choline pathway recorded for sac1 strains presents an intriguing paradox. The essence of the paradox is that, although hyperactivated CDP-choline pathway activity correlates with Sac1p-mediated bypass Sec14p, genetic inactivation of the CDP-choline pathway constitutes a recognized mechanism for bypass Sec14p (Cleves et al., 1991b). The DAG model illustrated in Figure 8 reconciles this contradiction in a simple manner. It posits that, al-
Although increased DAG production effects bypass Sec14p, the resultant consumption of DAG manifests itself in elevated CDP-choline pathway activity. The stimulation of the CDP-choline pathway in sac1 mutants raises the possibility that DAG availability helps set the baseline rate of metabolic flux through this pathway in yeast. Such a DAG effect could potentially be mediated by DAG stimulating the activity of CCTase, the rate-determining enzyme of the CDP-choline pathway.

Third, our results lend insight into the role of PtdIns-4-P accumulation in the sac1-mediated mechanism for bypass Sec14p. Because PLD-insufficient sac1 strains still accumulate high levels of this phosphoinositide (Figure 4, A and B), yet are incompetent for bypass Sec14p (Xie et al., 1998), we conclude that increased PtdIns-4-P is at best a contributing factor to bypass Sec14p. Indeed, the finding that PLD deficiency abolishes the bypass Sec14p phenotype of sac1 strains in the face of PtdIns-4-P accumulation suggests that excess PtdIns-4-P may contribute to bypass Sec14p by effecting a downstream activation of PLD (Figure 8). For example, PtdIns-4-P could modulate PLD activity indirectly by influencing the action of another protein whose function is to regulate PLD. The yeast oxysterol-binding protein homologue Kes1p and the BSD1 gene product represent candidate PLD regulators (Figure 8), because the bypass Sec14p growth phenotypes of kes1 and BSD1-124 mutants are also completely abolished by PLD insufficiency (Figure 4C). Kes1p is a particularly attractive candidate because it binds PtdIns-4-P, and Kes1p overproduction phenocopies PLD inactivation (our unpublished data). Interestingly, CDP-choline pathway mutations exhibit a significant PLD-independent component of their ability to suppress sec14 defects (Figure 4C). We attribute this PLD-independent component to reflect reduced rates of DAG consumption (Figure 8).

Fourth, we report insights into the mechanism that underlies the inositol auxotrophy of sac1 strains. We demonstrate that CDP-choline pathway activity contributes to this inositol requirement. Interference with the activity of this PtdCho biosynthetic pathway at any one of several points restores the ability of Δsac1 strains to grow in the absence of exogenous inositol (Figure 6B) at wild-type rates (Figure 7). Because sac1-associated inositol auxotrophy does not result in obvious defects in transcriptional regulation of INO1 (Figure 5, A and B), nor is it accompanied by the classical “inositol-less death” of ino1 mutants (Figure 7), we conclude that Sac1p deficiency results in an inability of cells to thrive on endogenously produced inositol. In that regard, the accumulation of PtdIns-4-P in sac1 strains indicates disruption of a substantial metabolic flux from PtdIns-4-P to PtdIns. This block in PtdIns production may contribute to the unusual ino-
sitol auxotrophy of sac1 strains in a manner that does not solely operate through CDP-choline pathway hyperactivation.

Finally, we emphasize the pleiotropic nature of the phospholipid metabolic alterations that accompany Sac1p dysfunction. Some of these are involved in mediating bypass Sec14p (i.e., accumulation of PtdIns-4-P and subsequent increases in PLD-mediated DAG production). We propose others to represent indirect correlates of the bypass Sec14p condition (e.g., accelerated CDP-choline pathway activity and reduced PtdSer levels). A continuing challenge in these analyses is the recognition of which alterations in phospholipid metabolism in bypass Sec14p mutants most directly mediate Sec14p-independent growth and Golgi secretory function in yeast.

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