The Yeast BSD2-1 Mutation Influences Both the Requirement for Phosphatidylinositol Transfer Protein Function and Derepression of Phospholipid Biosynthetic Gene Expression in Yeast

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ABSTRACT

The BSD2-1 allele renders Saccharomyces cerevisiae independent of its normally essential requirement for phosphatidylinositol transfer protein (Sec14p) in the stimulation of Golgi secretory function and cell viability. We now report that BSD2-1 yeast mutants also exhibit yet another phenotype, an inositol auxotrophy. We demonstrate that the basis for this Ino- phenotype is the inability of BSD2-1 strains to derepress transcription of INO1, the structural gene for the enzyme that catalyzes the committed step in de novo inositol biosynthesis in yeast. This constitutive repression of INO1 expression is mediated through specific inactivation of Ino2p, a factor required for transactivation of INO1 transcription, and we show that these transcriptional regulatory defects can be uncoupled from the “bypass Sec14p” phenotype of BSD2-1 strains. Finally, we present evidence that newly synthesized phosphatidylinositol is subject to accelerated turnover in BSD2-1 mutants and that prevention of this accelerated phosphatidylinositol turnover in turn negates suppression of Sec14p defects by BSD2-1. We propose that, in BSD2-1 strains, a product(s) generated by phosphatidylinositol turnover coordinatey modulates the activities of both the Sec14p/Golgi pathway and the pathway through which transcription of phospholipid biosynthetic genes is derepressed.

"Bypass Sec14p" alleles of the remaining four genes (i.e., BSR3, BSD1, BSD2 and SAC1) do not block PC synthesis via the CDP-choline pathway, and analysis of the products of these genes is expected to yield novel insights into both Sec14p function in vivo and the biochemical basis for the toxicity of CDP-choline pathway activity to yeast Golgi function. At present, the least characterized of these genes are BSD1 and BSD2. Each of these genes was identified on the basis of dominant “bypass Sec14p” alleles (CLEVES et al. 1991b), suggesting that the respective gene products exert a positive action with regard to Golgi secretory function.

In this report, we demonstrate that the BSD2-1 mutation not only evokes a suppression of sec14-associated Golgi defects, but that this allele also affects a tight inositol auxotrophy in yeast. These phenotypic similarities suggest the possibility that sec14 and BSD2-1-mediated suppression of sec14 growth and secretory defects share a common mechanism. We now report that the basis for the Ino- phenotype of BSD2-1 strains involves the potent inactivation of a specific transcription factor (Ino2p) required for expression of INO1, the structural gene for the enzyme that catalyzes the committed step in de novo inositol biosynthesis. Moreover, we demonstrate that these particular transcriptional regulatory defects in BSD2-1 mutants do not
We propose that these effects are mediated through the yeast strains used in this study are provided in Table 1. W, YPD and yeast minimal media have been described (SHERMAN et al. 1986). Viable cells were quantitated as the number of colonies formed per unit volume plated on solid YPD medium after 3 days at 26°C. Total cell numbers were determined by direct microscopic counting using a hemacytometer.

Assessment of the effects of inositol starvation on cell growth and viability: The appropriate yeast strains were grown to midlogarithmic phase in minimal medium. The cells were washed twice with double distilled H2O, washed once with minimal medium lacking inositol and choline (1°C medium), and resuspended in the same 1°C medium at a density of 1 x 10^6 cells/ml. At appropriate times postshift, an aliquot of cells was taken for the determination of total and viable cell numbers (FERNANDEZ et al. 1986). Viable cells were quantitated as the number of colonies formed per unit volume plated on solid YPD medium after 3 days at 26°C. Total cell numbers were determined by direct microscopic counting using a hemacytometer.

Phospholipid analyses: For steady-state [32P]-radio labeling experiments, the appropriate yeast strains were grown overnight in minimal medium lacking choline and subcultured into the same medium. The cultures were subsequently pretreated with [32P]orthophosphate (10 μCi/ml) and incubated for 20–24 hr at 26°C with shaking. For pulse-radio labeling experiments, cells were cultured in choline- and methionone-free minimal medium and challenged with [methyl-14C]-methionine (1 μCi/ml) for 50 min at 26°C. Procedures for

The genotypes of the yeast strains used in this study are provided in Table 1. YCp(INO2, UR3) and YEp(INO2, UR3) plasmids were constructed by subcloning INO2 as a 2.7-kb PstI-HindIII restriction fragment from pSClE (HOSAKA et al. 1994) into the YCplac33 and YEpplac195 vectors, respectively (Gietz and Sugino 1988). The YCp(SAC1, UR3) and YEp(SAC1, UR3) plasmids employed in these studies were described as pCTY134, respectively (Whitter et al. 1993). The YEp(pINO4, UR3) plasmid employed carries a 1.53-kb INO4 restriction fragment in Yep352 (HILL et al. 1986) and was generously provided by S. HENRY (Carnegie Mellon University, Pittsburgh, PA). The PNO2::CAT (chloramphenicol acetyltransferase) transcriptional fusion plasmid has been described (ASHBURNER and LOPES 1995) and was obtained from JOHN LOPES (Loyola University of Chicago, Maywood, IL).

Construction of a SEC14::INO1 translational fusion (PSEC14::INO1): A 1.8 kb INO1 coding region plus additional DNA downstream was amplified by the PCR using yeast genomic DNA as a template, and oligonucleotides 5'-GGGAATTCACGCGTGAATATCTTCTC-3' and 5'-CCGCATGCCTTTAAGCTTCTCC-3' as forward and reverse primers, respectively. Codons 3 and 4 (GAAGAT) of INO1 in the forward primer were converted to an SfI site (underlined) in a manner that did not alter Inolp primary sequence, and an SpI site (GCATGC) was engineered at the 5' end of the reverse primer. The INO1 PCR product was digested with Sfi and SpI, and the resultant fragment was inserted into pTZ19R vector to yield pRE91.

A 581-bp SEC14 promoter region was amplified by the PCR using pRE71 including the entire SEC14 gene as a template, and oligonucleotides 5'-GGGAATTCACGCGTGAATATCTTCTC-3' and 5'-CCGCATGCCTTTAAGCTTCTCC-3' as forward and reverse primers, respectively. An EcoRI site (GAATTG) engineered at the 5° end of the forward primer. In the reverse primer, codon 2 (GTG) of SEC14 was converted to codon 2 (ACA; reverse complement indicated in bold in the reverse primer sequence given above) of INO1, and the reverse primer was clamped by a Sfi site (GAGGCTC) engineered adjacent to codon 2 to facilitate construction of the fusion. The PCR product was digested with EcoRI and Sfi, and the resultant fragment was inserted into the corresponding sites of pRE91 to yield pRE90. The 2.2-kb EcoRI-SphI fragment of pRE90 was then subcloned into YCplac33 and YEpplac195 to yield the YCp(PSEC14::INO1) and YEp(PSEC14::INO1) plasmids, pCTY174 and pCTY175, respectively. The translational fusion resulted in expression of Inolp under the SEC14 transcriptional and translational control.

Assessment of the effects of inositol starvation on cell growth and viability: The appropriate yeast strains were grown to midlogarithmic phase in minimal medium. The cells were washed twice with double distilled H2O, washed once with minimal medium lacking inositol and choline (1°C medium), and resuspended in the same 1°C medium at a density of 1 x 10^6 cells/ml. At appropriate times postshift, an aliquot of cells was taken for the determination of total and viable cell numbers (FERNANDEZ et al. 1986). Viable cells were quantitated as the number of colonies formed per unit volume plated on solid YPD medium after 3 days at 26°C. Total cell numbers were determined by direct microscopic counting using a hemacytometer.

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were subsequently harvested and clarified cell-free extracts were grown overnight in I'-C' minimal medium. The cells were visualized and quantitated by phosphorimaging using prepared as described (ASHBURNER and LOPES 1995). Protein concentrations were determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard, and CAT activity was determined by enzymatic acetylation of [14C]-chloramphenicol (GORMAN et al. 1982). Radiolabeled chloramphenicol species were resolved by thin-layer chromatography using a chloroform-methanol (95:5) solvent system, and the secretion index was calculated from these values as described by SALAMA et al. (1990).

The appropriate yeast strains employed in this study are given.

### TABLE 1

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The complete genotypes of the yeast strains employed in this study are given.

Northern blot hybridization: The appropriate yeast strains were grown overnight in 1°C minimal medium. The cells were washed three times with 1°C minimal medium and incubated in 1°C medium for 4 hr at 30°C to permit derepression of transcription of phospholipid biosynthetic genes. Total RNA extraction, hybridization conditions, and probes used for specific detection of the CH02, OPI3, PIS1, CH01 and IN01 genes have been described in detail elsewhere (HOSAKA et al. 1994).

### Measurement of PIN02::CAT activity: Yeast strains carrying the PIN02::CAT reporter gene were grown in minimal medium to midlogarithmic phase. Cells were washed three times and either incubated in minimal medium supplemented with inositol and choline (1 mM final concentration each) or without inositol and choline for 4 hr at 25°C. Cells were subsequently harvested and clarified cell-free extracts prepared as described (ASHBURNER and LOPES 1995). Protein concentrations were determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard, and CAT activity was determined by enzymatic acetylation of [14C]-chloramphenicol (GORMAN et al. 1982). Radiolabeled chloramphenicol species were resolved by thin-layer chromatography using a chloroform-methanol (95:5) solvent system, and the secretion index was calculated from these values as described by SALAMA et al. (1990).

Invertase assays: The appropriate yeast strains grown in YPD or uracil-deficient minimal medium for plasmid maintenance (when appropriate), and subsequently subcultured into YPD medium for 3 hr at 25°C. Cells were washed and shifted to YPD + 0.1% glucose medium for 2 hr at 37°C. Total and extracellular invertase activities were then determined as described (BANKAITIS et al. 1989), and the secretion index was calculated from these values as described by SALAMA et al. (1990).

PI-turnover experiments: The appropriate yeast strains
were grown to midlogarithmic growth phase in defined minimal medium lacking inositol (1 mM medium). The cells were concentrated in a 1 mL volume and presented with [3H]-inositol (8 mCi/mL; Amersham Co.) for 30 min at 26°C with shaking. The radiolabeled cells were then washed three times in the same 1 mM minimal medium, resuspended in minimal medium containing inositol (1 mM) to initiate the chase, and aliquots (one-tenth volume) were taken at appropriate time points for determination of [3H]-inositol remaining in the phospholipid fraction. Phospholipids were extracted from yeast exactly as described (McGee et al. 1994a). Two-dimensional thin layer chromatography of the organic extracts demonstrated that, under these extraction conditions, PI was the predominant inositol phospholipid recovered from the organic phase, while other inositol phospholipids (e.g., inositol sphingolipids) separated into the aqueous phase. Thus, [3H]-PI was quantitated by direct liquid scintillation counting of the organic extracts.

Incorporation of [3H]-inositol into cells was measured by removing one-tenth of the radiolabeled culture, immobilizing the cells on glass fiber filters (0.5 μm diam), washing the filters with 30 volumes of ice-cold 2 mM inositol, and quantitating filter-bound radioactivity by scintillation counting. Viable cell numbers were determined by plating of serial dilutions of culture aliquots taken at the zero time point onto YPD plates, incubation of the plates at 26°C for 3 days, and counting of colony forming units.

RESULTS

Characterization of Ino- phenotype of BSD2-l mutants: We had previously reported that Δsac1 yeast strains not only experience a bypass of the normally essential requirement for Sec14p in Golgi secretory function and cell viability (Cleves et al. 1989), but that Δsac1 strains also exhibit an inositol auxotrophy (Wittmers et al. 1993). As those findings demonstrated that certain mechanisms of bypass suppression of Sec14p could manifest themselves in an abnormal inositol requirement for growth, we tested the remaining six classes of “bypass Sec14p” mutants for associated inositol auxotrophies. Such studies revealed that BSD2-l mutants also exhibited a strict requirement for exogenous inositol for growth. A comparison of the growth characteristics of wild-type, inol-l3 and BSD2-l strains on inositol-replete and inositol-free media is depicted in Figure 2A. The inol-l3 strain is defective in the activity of Inolp, the enzyme dedicated to conversion of glucose-6-phosphate to inositol-1-phosphate; an obligate intermediate in inositol biosynthesis in yeast (reviewed by Carman and Henry 1989). Whereas wild-type yeast do not require inositol for growth (these have de novo inositol biosynthetic capability), both the inol-l3 and BSD2-l strains exhibited clear Ino- phenotypes (Figure 2A). Interestingly, most heterozygous BSD2/BSD2-l diploid strains tested were Ino-, indicating that the Ino- phenotype associated with BSD2-l most often behaved as a recessive trait (see Figure 2A). However, we have always found BSD2-l to score as a dominant trait with regard to suppression of sec14 growth and secretary defects (Cleves et al. 1991b; not shown).

To further characterize the Ino- phenotype of BSD2-l strains, we compared the effects of inositol starvation on the viability of inol-l3 and BSD2-l strains. As shown in Figure 2B, both inol-l3 and BSD2-l strains maintained viability for 4 ± 2 hr after shift to inositol-free medium, after which time both strains exhibited inositol-less death. However, the kinetics of inositol-less death were more rapid for the inol-l3 strain. Whereas the inol-l3 mutant suffered a 1000-fold reduction in viability after a 24-hr period of inositol starvation, the BSD2-l strain experienced a 40- to 50-fold reduction in viability. The kinetics of inositol-less death for inol-l3 and BSD2-l strains recapitulated the differential rates of reduction in intracellular inositol pool sizes in these strains. While a 3-hr inositol starvation of wild-type cells had no effect on intracellular inositol pool size, such a starvation resulted in approximately a sixfold reduction in the estimated intracellular inositol pool size of the inol-l3 strain. However, as in the case of the wild-type strain, a 3-hr inositol starvation failed to elicit an appreciable reduction in the estimated inositol pool size of the BSD2-l mutant (not shown). These comparative pool measurements demonstrated that the more rapid kinetics of inositol-less death in BSD2-l strains (relative to BSD2-l mutants) correlated with a considerably sharper decline in intracellular inositol pool size in the inol-l3 strain.

Specific duplication of INO2 effects a suppression of the BSD2-l inositol auxotrophy: Characterization of the BSD2-gene has been precluded by our failure to recover BSD2-l clones on the basis of suppression of sec14 growth defects. The recessive inositol auxotrophy of BSD2-l strains provided the alternative strategy of recovering BSD2 clones by complementation of the Ino- phenotype of BSD2-l strains. Strain CTY479 (ura3-52, BSD2-l) was transformed with a yeast Ycp50 genomic DNA library and, from an estimated 14,000 Ura+ transformants screened, five Ino+ transformants were recovered. Plasmid linkage of the Ino+ phenotype was established by recovery of plasmid from each of the five Ino+ transformants, and the demonstration that the purified plasmids conferred an Ino+ phenotype to BSD2-l strains in retransformation experiments. Restriction mapping experiments indicated that these five plasmids contained overlapping inserts and one such plasmid (designated pCTY210) was characterized in detail. Nucleotide sequence analysis revealed that the Ino+ conferring gene carried by pCTY210 was identical to the yeast INO2 gene (not shown), the structural gene for a transcription factor required for expression of phospholipid biosynthetic genes (Nikoloff et al. 1992; Nikoloff and Henry 1994; see Figure 7). That the four remaining plasmids recovered from the Ino+ selection also carried INO2 was confirmed by our finding that these plasmids all complemented the Ino- phenotype associated with Δino2.

The possibility that INO2 and BSD2 represented the same gene was tested both by attempts to genetically inactivate BSD2-l by introduction of Δino2 alleles into BSD2-l sec14-l strains and by meiotic segregation analy-
Inositol Auxotrophy of BSD2-I Yeast

A +INO BSD2-1

WT +/BSD2-1 ino1-13

-INO

WT BSD2-1

+ /BSD2-1 ino1-13

Figure 2.—Inositol auxotrophy of BSD2-I mutants. (A) Yeast strains with the indicated relevant genotypes were streaked for isolation on either inositol-containing (+INO), or inositol-free minimal medium (--INO), and incubated at 26°C for 96 hr. (B) Effects of inositol starvation on cell viability. Yeast strains were grown in inositol supplemented minimal medium and shifted to inositol-free medium. Cells were harvested at the indicated time points postshift for determination of total and viable cell numbers (see MATERIALS AND METHODS). The total cell number and viable cell number data (open and closed symbols, respectively) are expressed as quotients relative to the corresponding values initially measured at time of shift. Symbols are as follows: ○ and ●, wild-type strain; △ and ▲, ino1-13 strain; □ and ■, BSD2-I strain. Yeast strains employed in these experiments included: CTY182 (wild type); CTY479 (BSD2-I); CTY417 (ino1-13); CTYD162 (MATα/MATα, BSD2-I/+).

ses. With regard to the former approach, we were unable to eliminate the dominant “bypass Sec14p” phenotype in BSD2-I/+ heterozygous diploids that had been transformed with an ino2A allele, as would be expected if IN02 and BSD2 were allelic (not shown). These data suggested a nonallelism between BSD2 and IN02. To confirm these results, integrative genetic mapping analyses were performed. A 2.5-kb HindIII-EcoRI fragment derived from the IN02 locus was subcloned into the yeast integration vector YIplac211. The resultant plasmid (pCTY211) was linearized at a unique BglII site within IN02 and integrated by homologous recombination into strain CTY1-1A (a BSD2-I sec14-1 ura3-52) with selection for Ura+ transformants. The resulting strain was mated to strain CTY212 (a BSD2-I sec14-1 ura3-52), the diploid sporulated, and meiotic progeny analyzed for linkage of Ura+ to the BSD2-I locus. The BSD2-I locus was recognized by its associated “bypass Sec14p” (i.e., Ts+) and Ino− phenotypes. Of 15 tetrads analyzed, a distribution of one parental ditype:six non-parental ditype:eight tetratype ascis was recorded, and this lack of cosegregation of the Ura+ and Ino−/“bypass Sec14p” phenotypes was indicative of nonlinkage between the BSD2-I and IN02 loci.

Neither increases in IN04 gene dosage nor opil mutations suppress the BSD2-I inositol auxotrophy: In addition to Ino2p, transcriptional regulation of yeast phospholipid (PL) biosynthetic genes is also responsive to the action of the IN04 and OPI1 gene products (Hoshizaki et al. 1990; White et al. 1991). The IN04 gene product is a transcription factor that partners with Ino2p, and this trans-activating complex binds the upstream activator site(s) of PL-biosynthetic genes (Ambroziak and Henry 1994; see Figure 7). The OPI1 gene product represses PL biosynthetic gene expression in the presence of inositol and, to a lesser extent, choline (Carman and Henry 1989). As shown in Figure 3, neither introduction of a YEp(IN04) plasmid nor of an opil::LEU2 allele restored an Ino+ phenotype to BSD2-I strains. This was in contrast to the ability of a YCP(IN02) plasmid to confer an Ino+ phenotype to the same BSD2-I strain. These collective data demonstrated a specificity of suppression of the BSD2-I inositol auxotrophy by duplication of IN02 that was neither recapitulated by substantial increases in IN04 dosage nor by genetic inactivation of the Opi1p-mediated repression of PL-biosynthetic gene expression. The opil::LEU2 result was unexpected as opil mutations have the effect of upregulating Ino2p expression (Ashburner and Lopes 1995), an effect also realized...
yeast grown in I°C medium. Inspection of the steady-state PL-profile of BSD2-1 strains indicated two clear abnormalities: the contribution of PMME and PDME to bulk membrane phospholipid composition was elevated some five- to eightfold in the BSD2-1 strain as compared with the wild-type strain and an approximate fourfold reduction in bulk membrane PC composition was recorded in the BSD2-1 strain relative to the wild-type strain. Introduction of YCp(INO2) into the BSD2-1 strain corrected the aberrant accumulation of undermethylated PC precursors that was diagnostic of defects in PL-methyltransferase activity (Figure 4A).

Second, the transcriptional derepression of PL-biosynthetic genes in BSD2-1 strains was directly monitored by preparing total RNA from yeast strains that had been grown in inositol- and choline-replete medium (I+C+; i.e., repressing conditions) and shifted for 4 hr to inositol- and choline-free medium (I+C-; i.e., nonrepressing conditions) to allow derepression of PL-biosynthetic gene expression. The INO1, CH02, OPI3, CH01, and PIS1 mRNAs were then evaluated by Northern blot analysis (see Figure 1 for assignment of the corresponding gene products to PL-biosynthetic reactions). As shown in Figure 4B, the BSD2-1 yeast strain was strongly defective in its ability to derepress transcription of the INO1, CH02, OPI3 and CH01 genes when compared with the wild-type strain. This inability to derepress transcription was most strikingly evident for INO1 whose expression was estimated to be reduced ≥50-fold relative to wild type under the experimental regimen employed. However, introduction of YCp(INO2) into the BSD2-1 strain fully restored the ability to derepress PL-biosynthetic gene transcription. As INO1 expression is required for inositol prototrophy, these data provided a full accounting for the Ino- phenotype of BSD2-1 strains. PIS1 expression served as a negative control for these experiments since transcription of this gene is not subject to repression by inositol and choline and, as expected, the BSD2-1 strain exhibited normal PIS1 expression (Figure 4B).

Third, to demonstrate that the Ino- phenotype of BSD2-1 strains was indeed the exclusive result of their inability to derepress INO1 transcription upon shift of such cells to inositol-free medium, we placed the INO1 gene under SEC14 promoter control (see MATERIALS AND METHODS) and introduced the PSEC14::INO1 construct on either low- (YCp) or high-copy (YEP) plasmids into inol-13 and BSD2-1 strains. SEC14 is constitutively transcribed to yield a moderately abundant message (BANKAITIS et al. 1989), and SEC14 expression is not subject to repression by inositol or choline (T. P. McGEE and V. A. BANKAITIS, unpublished data). As shown in Figure 4C, the YEp(PSEC14::INO1) plasmid restored inositol prototrophy to both inol-13 and BSD2-1 strains. This Ino- phenotype was dependent on the dosage of PSEC14::INO1, since neither inol-13 nor BSD2-1 strains carrying YCp(PSEC14::INO1) were able to grow on inositol-free medium (Figure 4C). We pre-

**FIGURE 3.** INO2 overexpression suppresses the inositol auxotrophy associated with BSD2-1. The appropriate yeast strains were streaked for isolation on uracil-deficient minimal medium that was either inositol-replete (+INO) or inositol-free (-INO), and incubated at 26°C for 96 hr. Yeast strains employed included: CTY811 (wild type); CTY860 (BSD2-1); CTY832 [BSD2-1/YCp(INO2)]; CTY878 [BSD2-1/YEp(INO4)]; CTY865 (BSD2-1 opi1::LEU2).

**BSD2-1 mutants are defective in the expression of phospholipid biosynthetic genes:** The demonstration that increased INO2 gene dosage corrects the Ino- phenotype of BSD2-1 strains suggested that BSD2-1 effected a defect in the transcription of Ino2p-activated PL-biosynthetic genes. This hypothesis was confirmed by three independent lines of evidence. First, the data indicated that BSD2-1 strains exhibited significant defects in PL-methyltransferase activities (CH02 and OPI3 genes products; Figure 1). These enzymes are dedicated to the conversion of phosphatidylethanolamine (PE) to PC, and the Ino2p/Ino4p-dependent expression of these enzymes is tightly repressed by inositol and choline in an Opilp-dependent fashion (WAEGTER et al. 1969; KODAKI and YAMASHITA 1987; CARMAN and HENRY 1989). As shown in Figure 4A, the undermethylated PC precursors phosphatidylmonomethylethanolamine (PMME) and phosphatidyltrimethylethanolamine (PDME) were detected only at very low steady-state levels in wild-type
sume that this dose-dependence reflects a difference in strength between the INO1 and SEC14 promoters.

**INO2 expression is itself defective in BSD2-1 mutants:** Ino2p is required for the Ino4p-independent trans-activation of its own structural gene (ASHBURNER and LOPES 1995), and we tested whether INO2 expression was also defective in BSD2-1 strains. INO2 expression was monitored via a sensitive reporter construct where the chloramphenicol acetyltransferase (CAT) structural gene is fused to the INO2 promoter (PI::NO2::CAT) (ASHBURNER and LOPES 1995). As shown in Table 2, INO2 promoter activity was reduced some 200-fold and 80-fold in the BSD2-1 strain, relative to the wild-type strain, when the strains were grown in what are normally derepressing conditions (I<sup>−</sup>C<sup>−</sup>) and repressing (I<sup>+</sup>C<sup>+</sup>) conditions, respectively. These data clearly demonstrated that BSD2-1 strains were profoundly defective in INO2 gene expression. A revealing result was obtained when INO2 promoter activity was measured in a BSD2-1 opi1::LEU2 strain. In that strain, the constitutive derepression of INO2 transcription normally associated with opi1 mutations was not observed; i.e., opi1::LEU2 failed to overcome the INO2 transcriptional defects imposed by BSD2-1 (Table 2). This epista-

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**Figure 4.**—**BSD2-1** mutants are defective in PL-biosynthetic activities whose expression is regulated by the INO2 gene product. (A) Phospholipid composition of bulk yeast membranes. The appropriate wild-type (solid bars), BSD2-1 (stippled bars), and BSD2-1/Ycp(INO2) strains (striped bars) were grown for 20–24 hr at 25°C in choline-free minimal medium supplemented with [35P]-orthophosphate (10 μCi/ml). Phospholipids were extracted, resolved, and quantitated as described in MATERIALS AND METHODS. The given values represent the average mole percentage of each indicated phospholipid species relative to total glycerophospholipid (n > 3). Strains employed were CTY182 (wild type), CTY479 (BSD2-1) and CTY832 [BSD2-1/Ycp(INO2)]. (B) Expression of phospholipid biosynthetic enzyme genes. The indicated strains (Lane 1, wild-type strain CTY182; Lane 2, isogenic BSD2-1 strain CTY479; Lane 3, isogenic BSD2-1, Ycp(INO2) strain CTY832) were cultured in minimal-medium containing inositol and subsequently shifted to inositol-free medium. After a 4-hr incubation at 26°C, total mRNA was extracted from each culture, resolved by formamide-agarose gel electrophoresis, and specifically probed for mRNAs derived from transcription of the indicated genes exactly as described (HOSAKA et al. 1994). Each individual mRNA species is identified by its corresponding structural gene designation at bottom. Samples were normalized by quantity of RNA loaded (10 μg RNA per lane). *PIS1*, whose expression is not regulated in response to inositol or choline, represented a loading control. (C) Growth of BSD2-1 and ino1-13 haploid strains expressing INO1 under SEC14 promoter control. Yeast strains with the indicated relevant genotypes were streaked for isolation on uracil-deficient minimal medium with (+INO) or without (−INO) inositol, and incubated at 26°C for 96 hr. The strains employed for these experiments included: CTY182 (wild type); CTY479 (BSD2-1); CTY832 (BSD2-1/Ycp(INO2)); CTY879 [BSD2-1/Ycp(PSEC14::INO1)]; CTY888 [ino1-13/Ycp(PSEC14::INO1)]; CTY857 [ino1-13/YEp(PSEC14::INO1)].
### Regulation of PIN02::CAT gene expression

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CAT activity</th>
<th>INO2 derepression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Gamma^-)</td>
<td>(\Gamma^+)</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.88 ± 0.26</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>BSD2-1</td>
<td>0.0125 ± 0.0003</td>
<td>0.0053 ± 0.0016</td>
</tr>
<tr>
<td>BSD2-1 (opi1::LEU2)</td>
<td>0.0128 ± 0.0009</td>
<td>0.0036 ± 0.0016</td>
</tr>
<tr>
<td>(ino2::TRP1)</td>
<td>0.0067 ± 0.0005</td>
<td>0.0067 ± 0.0007</td>
</tr>
</tbody>
</table>

The PIN02::CAT construct was integrated at the GAL4 locus of the following strains: CTY182 (wild type), CTY479 (BSD2-1), CTY866 (BSD2-1 \(opi1::LEU2\)) and CTY459 (\(ino2::TRP1\)). Chloramphenicol acetyltransferase (CAT) activity is expressed as percent conversion of chloramphenicol to the acetylated form/mg protein/hr. Values represent averages from three independent experiments. Cells were cultured either in inositol and choline-free medium (\(\Gamma^-\)), or in inositol and choline-replete medium (1 mM final concentration for inositol and choline; \(\Gamma^+\)). IN02 transcriptional derepression ratios for these strains were calculated as the quotient of averaged CAT activities measured in \(\Gamma^-\) medium divided by averaged CAT activities measured in \(\Gamma^+\) medium.

The \(ino2::LEU2\) phenotype of BSD2-1 strains are independent: We had previously established that inactivation of the CDP-choline pathway for PC biosynthesis, but not the PE-methylation pathway, bypasses the cellular requirement for Sec14p function (CLEVES et al. 1991b). The inability of BSD2-1 strains to derepress expression of PL-biosynthetic genes raised the important possibility that this global transcriptional defect defined the mechanism by which BSD2-1 effects “bypass of Sec14p”, particularly since structural genes of the CDP-choline pathway are also under Ino2p control. This possibility was excluded from further consideration by several lines of experimental evidence.

First, we tested whether overproduction of IN02 restored a Golgi secretory block to BSD2-1 \(sec14-I^p\) strains. As shown in Figure 5, introduction of a \(YCp(INO2)\) plasmid into a BSD2-1 \(sec14-I^p\) strain did not compromise the ability of such a strain to grow at 37\(^\circ\) relative to an isogenic strain that was not subject to increased IN02 dosage. Quantitation of the invertase secretory efficiencies of these strains at 37\(^\circ\) was consistent with the phenotypic data (Table 3). Introduction of a \(YEp(INO2)\) plasmid also had no effect on the efficiency with which BSD2-1 suppressed \(sec14\) growth and secretory defects (not shown).

Second, since genetic inactivation of the INO2 gene recapitulates the fundamental basis for the BSD2-1 inositol auxotrophy, we determined whether \(ino2\) mutations effected a suppression of \(sec14\) growth and secretory defects. Both phenotypic and biochemical analyses demonstrated that the \(ino2::LEU2\) allele did not rescue growth of \(sec14-I^p\) strains at 37\(^\circ\) (Figure 5A) and that this \(ino2\) disruption allele did not significantly alleviate the \(sec14-I^p\) Golgi secretory block (Table 3). Similarly, both \(ino4\) and \(opi1\) mutations failed to suppress \(sec14\) growth and secretory defects (not shown).

Finally, we tested the growth properties of a \(sec14-I^p\) \(ino2::LEU2\) strain carrying \(YEp(SEC14::INO1)\). As this strain expresses INO1 in an Ino2p/Ino4p-independent fashion, it can be grown on either \(\Gamma^+\) medium to assess any effects of growth medium on suppression.

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**FIGURE 5.** —INO2 is irrelevant to the bypass Sec14p phenotype of BSD2-1 mutants. Yeast strains of the indicated genotypes were streaked and incubated on YPD medium at 26\(^\circ\) or 37\(^\circ\), as indicated, for 48 hr.
Such a strain was unable to grow at 37°C regardless of which growth medium was employed (not shown); indicating that the failure of ino2::LEU2 to suppress secl4 defects was independent of the inositol and choline content of the growth medium.

**BSD2-1 yeast strains exhibit an accelerated rate of turnover for nascent bulk membrane PI:** A pulse-chase strategy was employed to assess the effects of BSD2-1 on bulk PI stability in yeast (see MATERIALS AND METHODS). In these experiments, the strains employed carried a YCP(INO2) plasmid so that the BSD2-1 strain could be cultured in the inositol-free medium employed for the pulse-radiolabeling. As shown in Figure 6A, wild-type strains exhibited a half-life for PI of 4.9 ± 0.3 hr under the conditions employed. The BSD2-1 strain, however, exhibited a significantly accelerated rate of bulk membrane PI turnover as evidenced by the demonstration that PI half-life in that mutant was only 2.5 ± 0.6 hr; a 42% reduction in bulk PI stability relative to wild-type yeast. This accelerated PI turnover in BSD2-1 strains was not reflected in measurable alterations in inositol sphingolipid metabolism as BSD2-1 strains exhibit wild-type sphingolipid composition and content as determined by pulse- and steady-state radiolabeling experiments (not shown).

We also attempted to determine the stability of PC synthesized by the CDP-choline pathway in wild-type vs. BSD2-1 strains using a [14C]-choline pulse-radiolabeling regimen. However, we found this PC pool to be very stable in both wild-type and BSD2-1 strains as we were unable to detect any significant turnover in a 24-hr chase period in either case (not shown). Thus, while we cannot make a strong conclusion regarding the effect of BSD2-1 on PC stability, it does not appear that BSD2-1 exerts an obvious destabilization of that particular PC pool.

Previous studies suggested the possibility that the yeast Sactp functions to prevent inappropriate turnover of inositol phospholipids (particularly PI) in cells (CLEVES et al. 1991a; WHITTERS et al. 1993). As loss of Sactp function results in phenotypes that mimic those associated with the dominant BSD2-1 mutation (i.e., bypass suppression of secl4 defects and an Ino- phenotype; CLEVES et al. 1989; WHITTERS et al. 1993), we considered the possibility that overproduction of Sactp might increase the stability of PI in BSD2-1 strains. Again, the strains employed in these experiments carried a YCP(INO2) plasmid (to facilitate culture of the BSD2-1 strain in the inositol-free medium employed for the pulse-radiolabeling) and Sactp overproduction (ca. 15-fold; WHITTERS et al. 1993) was driven by a YEP(SAC1, HIS3) plasmid. As shown in Figure 6A, overproduction of Sactp significantly depressed the rate of bulk membrane PI turnover in the BSD2-1 strain as it increased the half-life of PI from 2.5 ± 0.6 hr to a value similar to that measured for wild-type strains. Overproduction of Sactp in otherwise wild-type cells did not significantly affect the stability of bulk membrane PI, however (Figure 6A).

**Overproduction of SAC1 antagonizes suppression of secl4-1** by **BSD2-1, but not the associated Ino- phenotype:** Our finding that Sactp overproduction precluded the accelerated turnover of PI in BSD2-1 strains predicted that Sactp overproduction would reimpede secl4-associated growth and secretory defects in BSD2-1 strains if accelerated PI turnover lay at the heart of the mechanism of BSD2-1-mediated suppression of secl4 defects. The data demonstrating phenotypic fulfillment of this prediction are shown in Figure 6B. A BSD2-1 secl4-1 strain transformed with a YEps(SAC1) plasmid failed to grow at the secl4-1-restrictive temperature of 37°C whereas the same strain transformed with a YCP(SAC1) plasmid exhibited wild-type growth characteristics at this temperature. As expected, the BSD2-1 secl4-1/YEP(SAC1) strain exhibited reestablishment of the secl4 secretory block at its restrictive temperature of 37°C whereas the isogenic YCP(SAC1) partner exhibited wild-type secretory capacity (Table 3). These collective data suggest that Sactp and the BSD2-1 gene product have the capacity to interface with the same pathway for suppression of secl4 defects and that this pathway likely involves accelerated PI turnover. The antagonistic effect of Sactp overproduction on suppression of secl4 growth and secretory defects was specific to BSD2-1 mutants as introduction of YEps(SAC1) into secl4-1 strains carrying each of the other five classes of "bypass Sec14p" mutations (i.e., ekl1, pctl1, cpl1, bsr3, and BSD1; CLEVES et al. 1991b; Mcgee et al. 1994a) did not measurably affect the efficiency with which these suppressed secl4-1 growth and secretory defects (not shown). Simi-
overproduction of Sac1p in wild-type yeast strains also was without effect with regard to cell growth and secretory capability.

Finally, Sac1p overproduction failed to restore inositol prototrophy to BSD2-I strains (Figure 6C). This finding suggests that the molecular basis for the inositol auxotrophy of *sac1* strains is likely distinct from that which underlies the Ino" phenotype of BSD2-I strains. Indeed, in the *sac1* case, the Ino" phenotype is a function of the activity of the CDP-choline pathway for PC biosynthesis and is associated with alterations in inositol sphingolipid metabolism (B. G. Kearns, T. P. McGee and V. A. Bankaitis, unpublished data). The Ino" phenotype of BSD2-I strains is related to neither (not shown).
**Inositol Auxotrophy of BSD2-1 Yeast**

**FIGURE 7.**—The involvement of the BSD2-1 gene product in both the Sec14p pathway and the pathway for transcriptional derepression of phospholipid-biosynthetic genes. In model A, the BSD2-1 gene product functions to generate two signals each specific for the Sec14p and transcriptional signaling pathways, respectively. This scenario is consistent with the involvement of a phospholipase that hydrolyzes PI to generate two products e.g., diacylglycerol (DAG) or phosphatidic acid (PA) and a soluble inositol or inositol derivative) that independently interface with the Sec14p- and Ino2p-dependent pathways. Model A suffers from the fact that, while inositol has been established to be a potent repressor of Ino2p-dependent gene expression, this inositol effect is obligately mediated through Opi1p, and BSD2-1 exerts its transcriptional repression effects in an Opi1p-independent manner (see text, Table 2). A second alternative is offered by model B. In this model, action of the BSD2-1 gene product is proposed to generate a single active product of PI turnover (e.g., DAG or PA, or a derivative thereof), which ultimately affects a coordinate influence on the Sec14p- and Ino2p-dependent pathway function, respectively. Such an intervention into the latter pathway (perhaps via lipid-regulated kinases) does not require involvement of Opi1p. Finally, we cannot yet exclude a model where BSD2-1 strains exhibit altered transcriptional activity in a regulatory network, or networks, that independently affects both INO2 expression and of an activity that participates in the Sec14p pathway for Golgi secretory function. The 9-bp DNA sequence that is bound by the Ino2p/Ino4p complex, and is the &-acting element through which Ino2p/Ino4p-mediated regulation of transcription is exerted, is designated as UASINO.

**DISCUSSION**

The data clearly show that the fundamental basis of the Ino- phenotype of BSD2-1 strains is their inability to derepress transcription of the INO1 gene, whose product is obligatorily required for de novo inositol biosynthesis when inositol is removed from the growth medium. The evidence to this effect includes: the finding that subtle overexpression of the transcriptional activator protein Ino2p was sufficient to effectively suppress the Ino- phenotype of BSD2-1 mutants (Figure 3); Northern analyses demonstrating that BSD2-1 strains were incapable of derepressing the Ino2p-dependent transcription of phospholipid biosynthetic genes (i.e., INO1) upon shift to inositol-free medium unless Ino2p was overproduced (Figure 4B); and the demonstration that expression of INO1 from an Ino2p-independent promoter rescued the inositol auxotrophy of BSD2-1 strains (Figure 4C). The INO1 transcriptional defect was, at least in part, the result of the inability of Ino2p to autoactivate transcription of its own structural gene (Table 2).

Several additional points of interest were raised by these data. First, it is clear that the responsible defect was limited to Ino2p dysfunction as increased dosage of the structural gene for Ino4p (the transcription factor that partners with Ino2p to form the Ino2p/Ino4p trans-activator for INO1 expression) failed to suppress the BSD2-1-associated Ino- phenotype (Figure 3). These data identify Ino2p as the subunit of the Ino2p/Ino4p trans-activating complex through which the BSD2-1-associated misregulation of PL-biosynthetic gene expression was exerted. Second, we found that genetic inactivation of OPI1, which encodes a negative regulator of INO1 gene expression, also failed to recapitulate the suppression of the BSD2-1 Ino- phenotype observed upon Ino2p overproduction (Figure 3). As the BSD2-1 defect in INO2 transcription was epistatic to the constitutive elevation in INO2 transcription normally effected by opil mutations (Table 2; ASHBURNER and LOPES 1995), the data indicate that BSD2-1 strains imposed an Opi1p-independent inactivation of Ino2p. This is of interest because, as Opi1p is required for inositol-mediated repression of INO1 and INO2 transcription in an as yet undetermined manner (CARMAN and HENRY 1989; ASHBURNER and LOPES 1995), BSD2-1 strains likely inactivate Ino2p via an inositol-independent mechanism (see Figure 7). Our current understanding of transcriptional regulation of phospholipid biosynthetic enzyme expression in yeast makes no provision for such an Opi1p-independent regulation of Ino2p function.
What is the relationship between the Ino- and "bypass Sec14p" phenotypes of BSD2-I strains? The results obtained clearly indicate that these phenotypes are genetically separable as overproduction of Ino2p effected a complete correction of the Ino- phenotype, but had no effect on either phenotypic suppression of sec14 defects (Figure 5) or on biochemical suppression of sec14-associated secretory defects (Table 3). Moreover, a genetic recapitulation of defects in transcriptional derepression of INO1 and other structural genes for PL-biosynthetic enzymes (i.e., by disruption of the INO2 and INO4 genes) also failed to evoke either a phenotypic or a biochemical suppression of sec14 Golgi secretory defects (Figure 5, Table 3; see above). This demonstrates that suppression of sec14 defects by BSD2-I is not executed through a global misregulation of PL-biosynthetic genes.

The demonstration that BSD2-I strains exhibited accelerated PI turnover (Figure 6A), when coupled with the observation that both the accelerated turnover of PI and BSD2-I-mediated suppression of sec14 defects were specifically sensitive to overproduction of Sac1p (Figure 6, A and B), argues that accelerated PI turnover constitutes the mechanism by which BSD2-I effects bypass of the Sec14p requirement. This conclusion is in accord with the demonstration that expression of a mammalian PI/PC-transfer protein can phenotypically rescue the growth and secretory defects of sec14-I" yeast strains, and that the PI-transfer activity of mammalian PI/PC-transfer protein is essential for this phenotypic rescue (SKINNER et al. 1993; ALB et al. 1995). Moreover, the data suggest that a product(s) generated from PI 9256853), respectively, rescue the growth and secretory defects of sec14-I" yeast strains of yeast fails to effect rescue of the sec14-I" secretory block, challenge of such strains with a short chain diacylglycerol does evoke a partial suppression of the sec14-I" secretory block (not shown). This pharmacological rescue is not sufficiently powerful to phenotypically rescue sec14-I" growth defects, however, and the significance of these effects remains a matter of investigation. Nevertheless, the notion that exit of secretory proteins from the Golgi requires a sufficient membrane pool of lipid precursor (e.g., DAG) to stimulate transport vesicle biogenesis raises the possibility that the specific toxicity of CDP-choline pathway activity to yeast Golgi function may be related to inappropriate consumption of such a lipid precursor pool (the CDP-choline pathway directly consumes DAG; see Figure 1) than to elevated Golgi PC content per se.

Two general, and not necessarily mutually exclusive, models can account for these various effects in a manner that links the independent "bypass Sec14p" and Ino-" phenotypes of BSD2-I mutants to a common event influenced by the BSD2-I gene product (Figure 7). First, the data are consistent with a model where BSD2-I strains exhibit altered transcriptional activity in a regulatory network, or networks, that independently affects both INO2 expression and activity of the Sec14p pathway for Golgi secretory function. Alternatively, BSD2-I strains might experience an inappropriately amplified signal transduction cascade that independently interfaces with the pathways for INO2 derepression and regulation of the Sec14p pathway (Figure 7). A prediction of this latter model is that the BSD2-I gene product may either be a hyperactivated phospholipase or that it may be involved in effecting the activation of a phospholipase. Thus, the linkage of the signal transduction to the INO2 transcriptional derepression pathway might occur through posttranslational modulation of transcription factor function (e.g., through posttranslational regulation of Ino2p activity via lipid-activated protein kinase activity), while the effect on the Sec14p pathway might not exhibit a transcriptional basis. The concept that a phospholipase could effect “bypass Sec14p” at Golgi membranes is an attractive one given that phospholipase D and phosphoinositide metabolism may play critical roles in membrane trafficking reactions (CLEVES et al. 1991a; BROWN et al. 1993; COCKROFT et al. 1994; HAY et al. 1995; LISCOTITCH and CANTLEY 1995).

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LITERATURE CITED


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